# THE CHARACTERISATION OF THE OVINE SKIN RESPONSE TO ORF VIRUS INFECTION

**Thesis submitted for the Degree of Doctor of Philosophy**

**by**

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This thesis is dedicated to my parents for their love, support and tolerance !

## DECLARATION

I declare that all the work presented in this thesis has been composed and performed by myself. Contributions to the work of this thesis by colleagues are fully acknowledged in the text.

This work has not been, and is not currently being submitted for candidature for any other degree.

Andrea Lear.

# TABLE OF CONTENTS

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# ABBREVIATIONS USED IN TEXT

| | |
|---|---|
| **AchE** | = acetylcholinesterase |
| **AIDS** | = acquired immunodefficiency syndrome |
| **AMV** | = avian myeloblastosis virus |
| **AP** | = alkaline phosphatase |
| **APC** | = antigen presenting cell |
| **BSA** | = bovine serum albumin |
| **Ca** | = calcium ionophore |
| **CHO** | = Chinese hamster ovary |
| **CPE** | = cytopathic effect |
| **cpm** | = counts per minute |
| **DETC** | = dendritic epidermal T cell |
| **Dig** | = digoxygenin |
| **DMSO** | = dimethylsulphoxide |
| **DNA** | = deoxyribonucleic acid |
| **dNTP** | = deoxynucleoside triphosphate |
| **DTH** | = delayed type hypersensitivity |
| **EDTA** | = ethylenediaminotetra-acetic acid |
| **EGF** | = epidermal growth factor |
| **ELAM** | = endothelial lymphocyte adhesion molecule |
| **FBS** | = foetal bovine serum |
| **FCS** | = foetal calf serum |
| **FITC** | = fluoroscein isothiocyanate |
| **FL(M)** | = foetal lamb (muscle) |
| **G + C** | = guanine/cytosine |
| **G.I.T** | = guanidine isothiocyanate |
| **GM-CSF** | = granulocyte macrophage-colony stimulating factor |
| **h** | = hours |
| **HIV** | = human immunodefficiency virus |
| **HPV** | = human papilloma virus |

| | |
|---|---|
| **HRPO** | = horse radish peroxidase |
| **HSV (1)** | = herpes simplex virus |
| **ICAM-1** | = intracellular adhesion molecule-1 |
| **IFN-α (β, γ)** | = interferons α (β,γ) |
| **IL- 1 (2,3,4,6,8,10,12)** | = interleukins 1(2,3,4,6,8,10,12) |
| **ITR** | = inverted terminal repeat |
| **LC** | = Langerhan's cell |
| **LFA-1** | = lymphocyte function associated antigen |
| **min** | = minutes |
| **MHC class I or II** | = major histocompatibility complex class I or II |
| **MOI** | = multiplicity of infection |
| **mRNA** | = messenger RNA |
| **OPD** | = O-phenylene diamine |
| **PBS** | = phosphate buffered saline |
| **RNA** | = ribonucleic acid |
| **RT-PCR** | = reverse transcriptase polymerase chain reaction |
| **rvv** | = recombinant vaccinia virus |
| **SALT** | = skin associated lymphoid tissue |
| **SAPU** | = Scottish antibody production unit |
| **SDS** | = sodium dodecyl sulphate |
| **SFV** | = Semliki forest virus |
| **SIS** | = skin immune system |
| **SPF** | = specific pathogen free |
| **TCA** | = trichloroacetic acid |
| **TCID** | = tissue culture infectious dose |
| **TCR** | = T cell receptor |
| **TPC** | = trichloroacetic acid precipitable counts |
| **UV (A,B)** | = ultraviolet (A, B) |
| **VEGF** | = vascular endothelial growth factor |

# ABSTRACT

Orf is a highly contagious, eruptive skin disease of sheep and goats caused by a parapox virus. The virus enters through abrasions in the skin, where it replicates in the regenerating epidermal keratinocytes. Despite the generation of a specific antiviral response, orf virus reinfections can be established easily, although the lesions are milder and generally regress more rapidly than after primary exposure.

The cutaneous response to orf involves the formation of a dense network of MHC class II$^+$ dendritic cells at the lesion. The primary aim of this project was to characterise these dendritic cells and to identify the cytokines produced by orf infected keratinocytes *in vitro*, that might be involved in the accumulation of the dendritic cells *in vivo*.

*In vivo* studies of primary and secondary orf virus lesions identified the class II$^+$ dendritic cells to be a population of CD1$^-$ cells, which are also found within the dermis of normal ovine skin. A subpopulation of these cells also expressed the antigen, coagulation factor XIIIa. Factor XIIIa$^+$ dendritic cells comprised over half the dendritic cells seen in the network of a primary orf lesion but were only observed in small numbers, transiently, in the secondary orf lesion. All the dendritic cells lacked the expression of ovine macrophage markers. The proliferative response of the primary and secondary orf lesions also differed. High proliferative activity was observed in the epidermis and dermis in the primary response to orf but not in the secondary response. A few of the proliferating cells were identified as dendritic cells but it would appear that the dendritic cell network in both primary and secondary orf lesions does not arise by local cell division.

An *in vitro* model of orf virus infection of cultured ovine keratinocytes was established. The kinetics of infection and the ability of the cells to support viral replication was investigated. It was shown that viral antigen could be detected in the infected cells 12h post-infection, prior to the onset of the viral cytopathic effect (CPE) at 24h. The release of infectious virus particles into the culture supernatants was

observed only after the onset of CPE. Host cell protein synthesis was unaffected within the first 12 hours post-infection as measured by incorporation of $^{35}S$ methionine by the cells.

Analysis of cytokine production by cultured ovine keratinocytes showed that uninfected keratinocytes had the ability to secrete the cytokines Interleukin-8 (IL-8) and Granulocyte-macrophage-colony stimulating factor (GM-CSF). Infection of keratinocytes with orf virus stimulated the production of IL-8 but GM-CSF could not be detected at any timepoint post-infection, despite the high level of mRNA present within the infected cells. Exogenous ovine GM-CSF incubated with supernatants from infected cells rapidly disappeared. The addition of protease inhibitors to the supernatants did not prevent the clearance of GM-CSF and so degradation of GM-CSF by endogenous proteases produced by the infected cells is unlikely. The factors involved in the clearance of GM-CSF have not been identified.

The results presented in this thesis indicate that orf virus may possess virulence factors that interfere with the production of cytokines by keratinocytes. The significance of these *in vitro* studies in relation to *in vivo* observations of infection has yet to be determined but may relate to the unusual dendritic cell response in orf and to the incomplete immunity following infection.

# CHAPTER 1

## GENERAL INTRODUCTION

## 1.1. ORF VIRUS

### 1.1.1. Definition

Orf or contagious pustular dermatitis is a highly contagious, eruptive skin disease of sheep and goats. It is caused by a parapoxvirus which can also infect man. The condition has no systemic phase and is characterised by pustular lesions from which scabs develop. Little has been published on the development of immunity and it is known that protection against re-infections is incomplete and not long lasting, and the available, live attenuated vaccine is only of limited use (Buddle *et al.*, 1984, Kitching, 1987).

**Figure 1.1** *Orf lesions around the mouth and nares of a young lamb. Some of the lesions are highly proliferative, a feature which is more common to natural infections as opposed to those that are experimentally induced. Extensive lesions such as these interfere with feeding and lead to loss of condition.*

## 1.1.2. Epidemiology

Orf is a disease that is distributed worldwide wherever sheep and goats are raised. The disease occurs all year round but is most common during the spring and summer (Aynaud, 1923; Glover, 1928; Schmidt and Hardy, 1932). Although orf is regarded as primarily affecting domestic sheep and goats, it has also been reported to occur naturally in other animals (reviewed by Robinson and Lyttle, 1991) either in the wild, in captivity or as farmed domesticated species. A feature of the poxviruses is their lack of pathogenicity for the commonly used laboratory animals.

Orf virus is also transmissible to man, occurring usually as an occupational hazard through the direct contact of man with animals or animal products. Thus it is a disease associated with veterinary surgeons, farm workers, butchers and freezer workers (Purdy, 1955). The lesions caused are found most commonly on the hands or face and, although painful, they are seldom serious. In uncomplicated cases the lesion resolves 5-8 weeks after infection (Hodgson-Jones, 1951, Leavell *et al.*, 1968). The lesion that develops is quite different from the lesion in sheep and will be described in more detail below.

The traditional view of orf virus transmission is that the virus, shed into the environment in the form of infectious scab material, is very hardy and will persist in dust and wool which act as the source of new infections (Theiler, 1928; Glover, 1932; Boughton and Hardy, 1935). The virus then enters through epidermal abrasions, a prerequisite for establishing an infection, caused by grazing in rough pasture or by scratches from fencing materials contaminated with the virus. Thus natural infections are normally localised around the mouth and nose or above the hooves. Human infections are thought to occur in a similar manner through contact with infected animals or contaminated fomites such as rails and wool hooks, and the presence of wounds and abrasions on the arms and hands increase the risk of infection (Pask *et al.*, 1951). Person to person transmission of orf has also been reported (Lang, 1962).

Typically outbreaks of orf are seen in susceptible animals (usually lambs) on a property that does not vaccinate and, once the pasture becomes contaminated, it is difficult to eradicate, the shed scabs of each infected animal contributing to the environmental pool of infection. However this traditional view of transmission does not account for virus getting into previously clean premises and surviving between outbreaks. One explanation for the persistence of virus in infected premises is that orf virus can survive for long periods in dry preparations, especially in scabs (Glover, 1930; Livingston and Hardy, 1960; Leavell *et al.*, 1986; Jubb and Kennedy, 1970). Although such persistence could occur in areas where animals are housed, infectious virus is much less likely to be maintained in this way on pastures exposed to the elements. Exposure to ultraviolet (UV) radiation reduces the infectivity of orf virus (Manley, 1934; Sawhney, 1972) and Mckeever and Reid (1986) showed that wet weather conditions in Scotland are not conducive for scab borne viral infectivity indicating that the large number of orf virus outbreaks observed in the spring are unlikely to be caused by orf virus that had wintered in scabs in this country.

The method of transmission of orf virus into new, clean premises remains to be elucidated. It has been proposed that subclinical orf infections or animals suffering from chronic or trivial orf virus infections act as carriers (Greig *et al.*, 1984; Mckeever, 1984). At present there is still insufficient evidence to support this view. It is generally believed that the spread and maintenance of the infection is related to the resistant nature of the virions in the environment and the short-lived immunity to re-infection.

### 1.1.3. The clinical pathology of the disease

Natural orf infections are established in, and generally restricted to, the site of epidermal abrasions, commonly the lips, mouth and nose of lambs (see Figure 1.1) and udders of nursing ewes. The virus is highly epitheliotropic and the lesions produced are found generally on the epithelium and oral mucosa, although in some cases it may affect other tissues of the body (reviewed in Robinson and Balassu, 1981 and Watt, 1983). Application of a viral suspension to scarified skin is the established method of inducing orf experimentally (Glover, 1928; Wheeler and Cawley, 1956; Abdussalam,

1957, Kluge *et al.*, 1972). Skin lesions develop initially as reddening and swelling around the sites of inoculation and the lesions progress through the stages of papules, vesicles and pustules to scab formation. In uncomplicated cases the disease is afebrile and self limiting. Secondary papules, vesicles and pustules often develop so that the lesion involves 1-2 cm of skin surrounding the initial inoculation site. In a primary experimental infection scabs form within one week and are shed within 4-6 weeks without leaving a scar. Secondary infections are generally milder, the scab forms within 3-4 days and complete resolution of infection is seen by 14 days (Robinson and Balassu, 1981).

The effects of the disease on the animal are determined by the site and extent of the lesions. Lesions around the mouth and nose interfere with feeding, retarding the growth of young lambs. Teat lesions on the udders of nursing ewes can also interfere with the suckling of young lambs. Complications such as mastitis can arise as a result of opportunistic bacterial infections. Highly proliferative lesions that are not normally observed in experimental orf infections are more common in natural orf infections (see Figure 1.1). These proliferative papillomatous lesions form dense wart-like outgrowths that together with secondary complications, become ulcerative and necrotic without scab formation. The process of healing is delayed, resulting in much more persistent infections (Glover *et al.*, 1928; Newsan and Cross, 1931; Selbie, 1944; Darbyshire, 1961).

Orf virus infection of man produces a lesion that is significantly different from the lesion observed in sheep. Whilst the human lesion progresses through the macular, papular, vesicular and pustular stages seen in sheep, it is characterised by the dilation and the extensive proliferation of the vascular endothelium of the dermal capillaries. This results in a marked umbilication of the lesion that is not observed in sheep (reviewed by Yirrell and Vestey, 1994). The cellular components involved in the human response have not yet been identified.

### 1.1.4. Orf virus structure

Orf virus is a member of the genus parapoxvirus belonging to the family Poxviridae. The parapoxviruses are morphologically similar and can be distinguished from other poxvirus genera by 3 main criteria: they are 1) more cylindrical, 2) have a higher axial ratio and 3) the tubular threads on their outermost coat are arranged in a direct basket weave fashion rather than the random arrangement observed for the orthopoxviruses (see Figure 1.2). These viruses do however share a common antigens, such as the nucleoprotein antigen, with the other poxvirus genera (Webster, 1958; Woodroofe and Fenner, 1962).

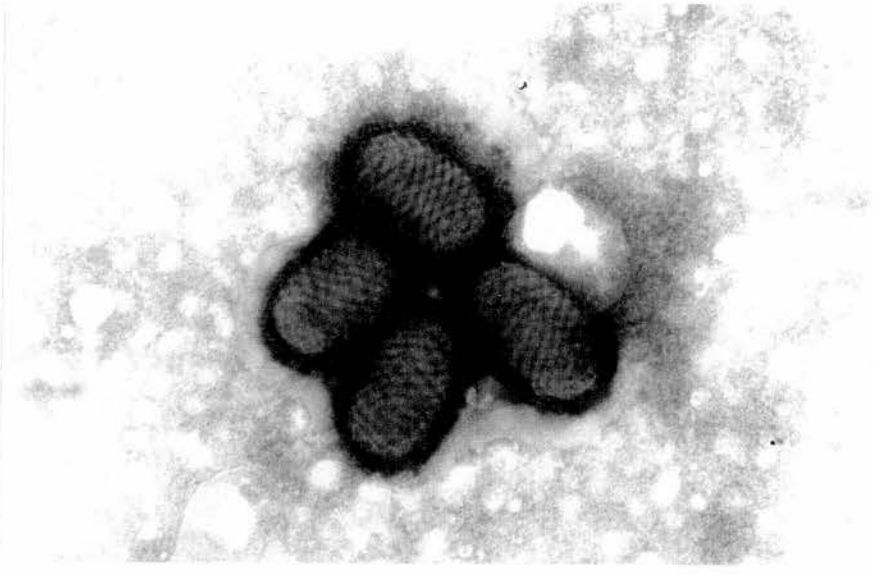


**Figure 1.2** *Electronmicrograph of negatively stained orf virus showing the outer tubular coat of the viral particles, the 'M' form.* **X 80,000.**

Orf virus is oval in outline with a long axis of 260nm on average and a short axis of 160nm with an axial ratio of 1.6 (Nagington and Horne, 1962; Abdussalam and Cosslett, 1957). The viral coat comprises an inner and an outer envelope and cell culture isolates of orf virus appear to have an additional membrane which, by analogy

with vaccinia, is thought to be derived from the host cell Golgi body (Rosenbusch and Reed, 1983; Mitchner, 1969). Electron microscopy of negatively stained orf virus particles reveals two forms of orf virus; the 'M' or mulberry form, where the outer tubular coat of the virus is highlighted (see Figure 1.2) or the 'C' or clear form, where the stain has penetrated the viral coat to reveal the capsule of the virus (Nagington and Horne, 1962; Knocke, 1962; Buttner *et al.*, 1964; Nagington *et al.*, 1964).

The internal structure of orf virus is similar to other poxviruses (Knocke 1962; Nagington and Horne, 1962; Peters *et al.*, 1964). It consists of an internal S shaped tubular structure surrounded by a lipoprotein envelope (Mitchner *et al.*, 1969). Orf virus contains linear double stranded DNA of approximately 137kb in size and in common with the other parapoxviruses has a high guanine and cytosine (G + C) content of up to 63% (Wittek *et al.*, 1979). Like the DNA of vaccinia virus and papular stomatitis virus, orf virus DNA has crosslinked ends.

### 1.1.5. Strain variation

Until relatively recently it was generally perceived that only one strain of orf virus exists as cross protection trials between isolates from the UK, USA, France and Australia did not show any strain variation (Glover, 1932 and 1933). The results obtained from the different groups were however quite variable, indicating the unreliable nature of the tests used. A wide range of methods employed by various workers to measure antigenic variation between orf virus strains (reviewed by Robinson and Balassu, 1981) also gave conflicting evidence. Buddle *et al.*, (1984) were able to organise different viral isolates into groups based on the electrophoretic analysis of the structural polypeptides, whereas they had been unable to find differences using serum neutralisation tests. More recent studies employing restriction endonuclease analysis of orf virus DNA have indicated that there is considerable heterogeneity between isolates, which could be related to antigenic diversity (Robinson *et al.*, 1987). The biological significance of this diversity is not known. Antigenically the other poxviruses; bovine papular stomatitis virus, milker's nodule virus and pseudocowpox virus appear to be closely related to each other and to orf

virus, which would support the view that biologically significant diversity of orf virus isolates is unlikely.

### 1.1.6. Vaccination

Orf is the only parapoxvirus disease where vaccination is practised routinely as a control measure. Vaccination is by the use of a live virus vaccine in essentially the same manner as that described by Aynaud (1923). The animal is inoculated at 3-4 weeks of age on the leg with unattenuated virus (Aynaud, 1923; Glover, 1928; Schmidt, 1962; Howarth, 1929; Boughton and Hardy, 1935). A typical orf lesion occurs at the site of the inoculation and after 12-13 days the animal is "immune" to reinfection (Robertson, 1976). The animals are protected for approximately 5 months after which infections can be contracted but they tend to be milder (Roberston, 1976). The primary aim of vaccination is to protect the animal from developing the severe disease during the crucial early growth stage or passing infection to the udder of the ewe thereby reducing production losses in the lamb-meat industry. The disadvantages of the vaccination protocol are that the vaccines are not fully protective (Buddle *et al.*, 1984) and, as the vaccine is a live virus preparation, the scabs that are formed following vaccination contaminate the environment.

### 1.1.7. The orf virus genome

Most of the work characterising pox virus genomes has been carried out on the orthopoxviruses, particularly vaccinia virus. It would appear that all pox viruses are related even at the genome level. However the distinctive morphology of the virions and the high G+C content of the genome of the parapoxviruses have set them apart from the orthopoxviruses (Robinson and Balassu, 1981; Robinson and Lyttle, 1991). Vaccinia virus has a genome of 199kb and a G + C content of 36% compared with the genome of orf virus which is 60kb smaller and has a higher G + C content of 63%. Vaccinia virus has been extensively characterised and whilst orf and vaccinia differ in their G + C content, recent studies have shown that, if their gene sequences are translated into peptide sequences and compared, they share a number of putative proteins in common (Fleming *et al.*, 1993). Furthermore it was found that some of the

proteins were in the same relative positions allowing a tentative alignment of the two genomes (see Figure 1.3, Fleming *et al.,* 1993).

Selected regions of the orf virus genome have been sequenced. A 4kb region at the left end of the genome which includes the inverted terminal repeat (ITR) and 1.5kb of unique sequence has revealed three genes transcribed early in infection, two of which are unique to orf virus (Fraser *et al.,*1990, Fleming *et al.,*1991, 1992). The third gene shows homology at the amino acid level to a UTPase-like gene in vaccinia virus (Mercer *et al.,*1989; Slabaugh and Roseman, 1989; McGeoch, 1990). Sequencing of another fragment revealed a 10kDa polypeptide with homology to the 14k "fusion" gene of vaccinia virus (Rodriguez and Esteban, 1987; Rodriguez *et al.,*1987, Naase *et al.,*1991). The relative position on the genome and the orientation of the dUTPase-like and 14kDa-like genes are similar in the two viruses suggesting that despite the divergence in G + C content of the genomes and the differences in morphology, the general arrangement of genes may have been conserved between orf virus and vaccinia virus. A recent study has identified an orf virus homologue of the vaccinia virus gene encoding the major envelope antigen P37K (Sullivan *et al.,*1994). The protein produced also shows homology to the proteins encoded by molluscum contagiosum virus and another encoded by fowlpox virus. When the orf virus gene was expressed it was found to be one of a limited number of orf virus proteins to which sheep mount a strong antibody response following a natural infection and which stimulate lymphocytes derived from lymph nodes draining a site of infection.

Vaccinia virus gene products have recently been identified that interfere specifically with a number of host defence mechanisms (discussed in more detail later). Some of these genes confer resistance to the cytokines interferon (IFN) and interleukin-1. (IL-1) (reviewed by Smith, 1993). It would be of great interest therefore if these genes are conserved within the orf virus genome. However it is the genes closest to the termini of the vaccinia virus genome that are the determinants of virus virulence, tissue specificity and host range (Pickup *et al.,*1984; Kowtal and Moss, 1988, Boursnell *et al.,*1988, Perkus *et al.,*1991) and when vaccinia is aligned with orf virus, the termini containing these genes lie outwith the orf virus genome (Figure 1.3).

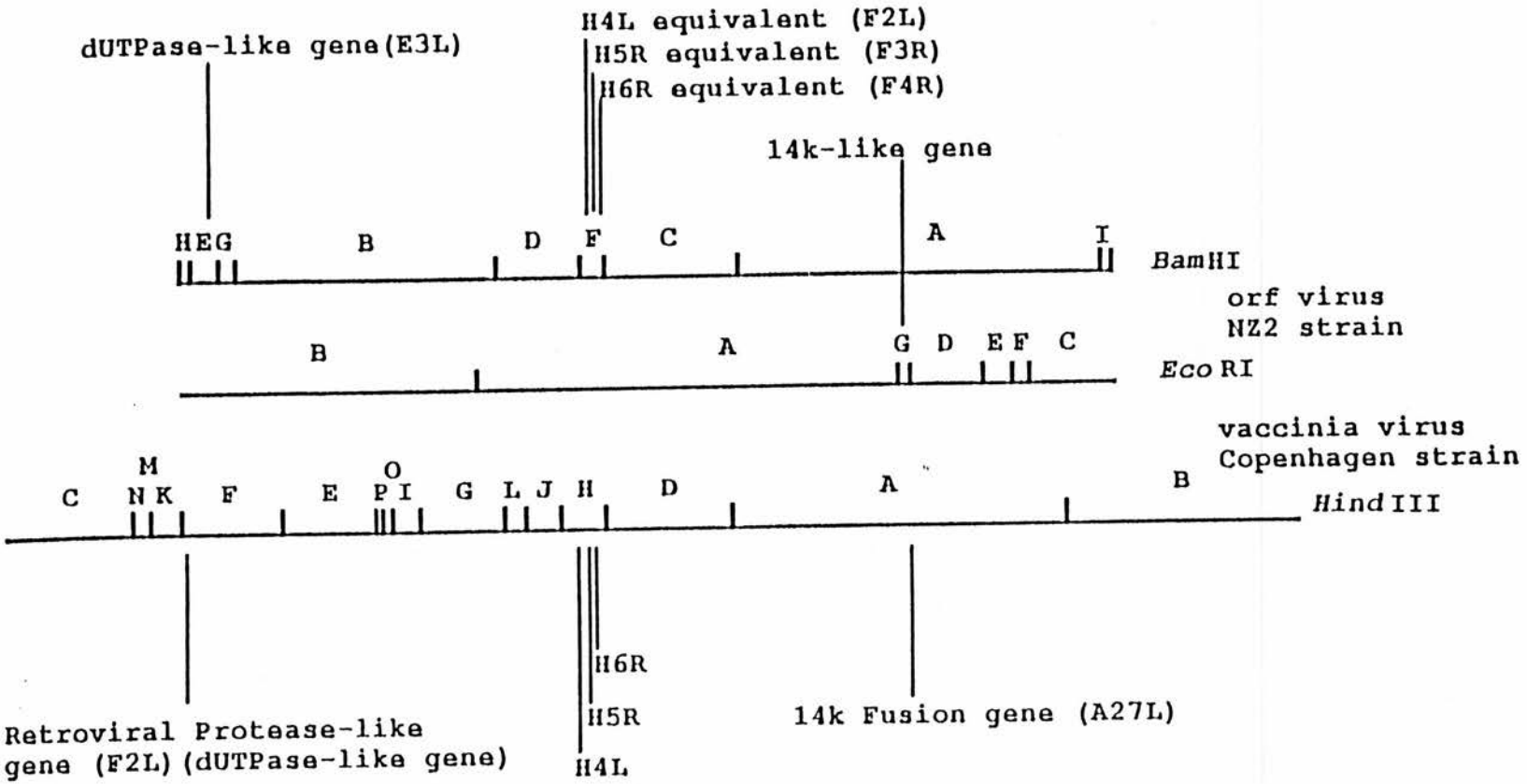



**Figure 1.3** *Alignments of orf virus and vaccinia virus genomes showing the positions of homologous genes (Fleming et al., 1993). Retroviral protease-like gene (F2L)(dUTPase-like gene), Slabaugh and Roseman (1989); dUTPase-like gene (E3L), Mercer et al (1984); H4L, H5R, H6R, Rosel et al., (1986), Goebel et al., (1990); 14K fusion gene Naase et al., (1991).*

## 1.1.8. Immune responses to orf virus infection

Orf virus has the ability to re-infect its host resulting in the development of clinical lesions. The re-infected animal however suffers a milder, more rapid course of infection. It is generally accepted that orf virus does confer immunity on the host. The nature and duration of this immunity remains controversial. It was suggested that orf virus induces lifelong and complete protection against re-infection,(Aynaud, 1923; Altara, 1925; Jacotot, 1926; Glover, 1928; Abdussalam, 1958; Hardy, 1964; Jubb *et al.*,1985) but there is ample evidence to the contrary. The susceptibility of the recovered animals to re-infection and the fact that vaccinated animals are also susceptible to infection would confirm the view that orf virus confers only partial immunity on the animal (Schmidt, 1962 and 1967; Lopatnikov, 1968; Frerichs, 1980; Kovalev *et al.*,1971; Osman, 1976; Buddle, 1981; McKeever, 1986 ;McKeever *et al.*,1988). The duration of this immunity appears to be very variable. Secondary orf infections in humans have also been recorded within 14 months of the first, indicating that immunity in man may also be shortlived (Robinson and Petersen, 1983).

### *i). Humoral immunity*

Early studies showed that serum from immune animals was not protective (Aynaud, 1923) and, although neutralising antibody present in colostrum is passed on to lambs from their immune dams, it did not appear to be protective (Buddle and Pulford, 1984). This is consistent with reports that lambs born to immune dams are still fully susceptible to infection within the first few days of life (Boughton and Hardy, 1935; Schmidt, 1967; Richeter and Jansen, 1968). However in other experiments, the titre of neutralising antibody in colostrum appeared to correlate directly with the degree of protection transferred to the lambs (Poulain *et al.*,1972, Jan *et al.*,1978).

It was demonstrated, that following orf virus infection, viral neutralising, agglutinating, precipitating and complement fixing antibodies were produced (Buddle and Pulford, 1984; Poulain *et al.*,1972, Jan *et al.*,1978; Nagington and Whittle, 1961; Abdussalam, 1958; Schmidt; 1967). Circulating antibody can be detected as early as 6 days after inoculation and Western blotting showed that the antibody from infected

animals would bind to preparations of orf virus antigens (McKeever, 1987). Naturally infected animals with chronic lesions showed responses to at least 13 virion components, with some variation. Experimentally infected animals responded more consistently but only to 3 or 4 viral antigens, one of which was a 40kDa protein identified as the characteristic surface antigen. Despite the production of virus specific antibody however, there is no convincing evidence that humoral responses are involved in protection against infection with orf virus or in the resolution of the lesions as infections can be established in animals with pre-existing antibody. It seems more likely, therefore that recovery from the disease is the result of cell mediated immunity.

### *ii). Cell mediated immunity*

The first attempt to demonstrate the role of cell mediated immunity in orf virus infection was an adoptive transfer experiment carried out by Osman (1976). Naive sheep were inoculated with sensitised splenic and thymic cells from immune sheep in an attempt to transfer immunity to orf. However the sheep were not protected from orf and developed lesions that could not be distinguished from the controls. As identical twins were not used, the sheep were not major histocompatibility antigen matched and thus the experiment has received some criticism. Other studies successfully demonstrated the involvement of cell mediated immunity to orf virus by indirect macrophage inhibition tests, direct leucocyte migration inhibition and lymphocyte transformation tests (Maeda, 1979). Sheep previously exposed to orf virus demonstrated a delayed type hypersensitivity (DTH) reaction when injected intradermally with killed orf virus antigen (Buddle and Pulford, 1984). Furthermore this DTH provided a way of distinguishing between lambs that had been actively immunised and those that had been exposed to colostral antibodies, as only the immunised lambs gave a positive DTH response. Peripheral blood lymphocytes demonstrated proliferative activity to orf virus antigen that preceded the detection of specific antibodies in the blood (Yirrell *et al.*, 1989). This occurred approximately 7-14 days after primary exposure to infection and higher proliferative responses could

be detected following secondary infection although there was greater variation between individual sheep than in the primary resonse.

The conclusions to be drawn from these experiments suggest that the humoral and cellular responses to orf virus infection are typical of those following an acute cutaneous viral infection. However, they provide little in the way of information about the mechanisms of the protective response. More recently work has focused on the local cellular immune response as being important in the control of, and protection against, orf virus infections.

Before describing the local cutaneous response to orf virus, the cellular components of the skin immune system that are important in the generation of a local cutaneous immune response will be discussed.

## 1.2. THE SKIN IMMUNE SYSTEM (S.I.S)

The skin is the largest organ of the body and in addition to providing a protective barrier, it also carries out a number of important physiological and immunological functions. The skin contains a number of indigenous cell types that have the ability to initiate both local inflammatory and systemic immune reactions. The concept of skin associated lymphoid tissue (SALT) was introduced by Streilein (1978). Streilein used this term to describe the specialised immune mechanisms of the skin including 1. the recirculating population of T-lymphocytes that have skin seeking tendencies (epidermotropic T cells), 2. the dedicated antigen presenting cells of the epidermis (the Langerhans cells) and 3. the skin draining peripheral lymph nodes. Since then other immunologically active cell types and their mediators have been recognised within the skin and these cell types taken together with those comprising SALT, form an intricate and complex immune system known as the skin immune system (SIS) (Bos and Kapsenberg, 1986). These newly recognised cells include keratinocytes, mast cells, polymorphonuclear neutrophils, γδ-type T cells, indeterminate cells, veiled cells, vascular endothelium cells, afferent lymphatic endothelium and most recently the dendritic cells of the dermis.

The cellular components of SIS have been extensively reviewed by Bos and Kapsenberg (1986, 1993), Bos (1990) and the major cell types involved in cutaneous immunity have been highlighted and updated below.

### 1.2.1. The role of epidermal Langerhans/dendritic cells in cutaneous immunity

Dendritic cells originally described by Steinman *et al.*, (1979) are a system of bone marrow derived antigen presenting cells that are widely distributed throughout lymphoid and non-lymphoid tissues of the body (Table 1.1).

**Table 1.1** *The dendritic cell system-distribution and nomenclature*

| Tissue | Area | Name |
|---|---|---|
| **Non-lymphoid organs** | Skin | Epidermal Langerhans cells<br>Dermal dendritic cells |
| | Heart, Liver, Lung, Gut | Interstitial cells |
| **Lymphoid organs** | Spleen, Thymus | Interdigitating cells |
| | Lymph nodes - B cell area<br>- T cell area | Interdigitating cells<br>Follicular dendritic cells |

The dendritic cells from the different locations of the body all share characteristic features that distinguish them from other antigen presenting cells as they, 1) exhibit a typical dendritic and 'veiled' morphology, 2) constitutively express high levels of major histocompatibility (MHC) class II molecules on their surface, 3) lack or have comparatively low amounts of markers of the monocyte-macrophage lineage, 4) have a limited phagocytic potential in comparison with professional phagocytes and 5) show strong stimulatory capacity for resting T lymphocytes in proliferation assays, such as the mixed leucocyte reaction, with the ability to cluster in an antigen independent manner.

### *i). Langerhans cells as immature dendritic cells*

Langerhans cells (LC) are the best characterised of the dendritic cells. They represent the most peripheral outpost of the immune system and function as a critical link between the extracutaneous environment and the organism. The discovery of LC as an antigen presenting cell led to numerous productive experimental studies (reviewed by Romani and Schuler, 1992). Based on these studies the general consensus view on the function of the LC is that they are the primary antigen presenting cell of the skin. LC pick up antigen from the epidermis, process it and carry the antigen on the surface of the cell in association with MHC class II molecules. The antigen laden cells travel across the epidermal/dermal junction into the dermis and are then carried by the flow of the afferent lymph to the draining lymph node. Once in the lymph node, LC are believed to be the unique antigen presenting cells that can activate and prime naive, antigen specific T lymphocytes.

*In vivo* and *in vitro* studies have revealed significant phenotypical and functional differences between LC and the related lymphoid dendritic cells. Functionally, lymphoid dendritic cells are poor antigen processing cells (Kapsenberg *et al.*, 1991). However they are potent antigen presenting cells and have the capacity to activate resting lymphocytes *in vitro* and induce contact sensitisation *in vivo* (Cumberbatch *et al.*, 1991a). In contrast freshly isolated LC are very efficient at processing soluble protein antigens (Romani *et al.*, 1989) but they are relatively inefficient antigen presenting cells and fail to form stable clusters with T cells (Cumberbatch *et al.*, 1991b). It has been hypothesised that freshly isolated LC are the *in vitro* functional equivalents of the intra-epidermal LC, whereas cultured LC are equivalent to LC that have already migrated to the draining lymph node. This proposal contains the concept that the increase in LC antigen presenting cell function that occurs during culture parallels the differentiaton programme of LC that have migrated to the lymph node.

A comparison between the populations of freshly isolated LC and cultured LC has revealed phenotypical, morphological and functional differences that could be related to the specific roles of LC and lymphoid dendritic cells (reviewed by Romani and

Schuler1992). The main function of the LC is the uptake and processing of antigen. The presence of acidic organelles and the high cellular synthesis rate of MHC class II and the invariant chain (Brodsky, 1991; Becker, 1991) could be related to their efficiency at processing and presenting antigen. These properties are lost on culture (Streilein, 1989; Kampgen *et al.*, 1991) and replaced by the increased ability of the cell to present antigen in association with MHC class II and stimulate T cells. Associated with this new-found ability of the cell is an increase in the expression of MHC class II molecules (Romani *et al.*, 1989; Schuler and Steinmann; 1985; Shimada *et al.*, 1987; Teunissen *et al.*, 1990; Witmer-Pack *et al.*, 1988) and adhesion molecules involved in T cell interactions (Romani and Schuler, 1992), discussed in more detail below.

LC have very poor phagocytic activity compared with the professional phagocytes. Birbeck granules and CD1a surface antigen associated with LC have been implicated in antigen uptake (Hanau *et al.*, 1987). Birbeck granules are proposed to delay excess degradation of antigens and this, together with the slow turnover of the surface MHC class II molecules, is thought to ensure that antigen presentation can still occur in the time taken for LC to make contact with antigen-specific T cells (Kapsenberg *et al.*, 1990).

### *ii). Migration of LC*

The ability of LC to migrate is supported by a number of studies. Following skin sensitisation there is a gradual reduction in LC, an increase in afferent lymph veiled cells (many containing Birbeck granules) and an accumulation of dendritic cells in the lymph nodes (Hoefsmit *et al.*, 1982; Kripke, 1990; Knight *et al.*, 1985a and b). Following the topical application of the contact sensitiser fluoroscein isothiocyanate (FITC) to the skin of mice, dendritic cells were found in the draining lymph node that were carrying FITC and had the ability to stimulate FITC specific primary T cells (Macatonia *et al.*, 1987). *In vitro*, Larsen *et al.*, (1990) created an organ culture skin model and demonstrated the capacity of LC to leave the epidermis, accumulating as MHC class $II^+$ dendritic cells within the dermis and subsequently migrating out from the skin explant into the culture medium.

Collectively, these studies make it very likely that LC migrate from the epidermis to the lymph nodes. However the possibility that some of the migrating cells are dermal dendritic cells has to be considered and will be discussed later.

### *iii). Antigen presentation*

The primary site of sensitisation to antigen is the regional lymph node. There the antigen laden dendritic cell stimulates the T cells through the interaction of the T cell receptor TCR/CD3 complex on the T cell specific for the MHC/peptide complex on the surface of the dendritic cell. These T cells are then stimulated to proliferate and produce cytokines. A unique feature of dendritic cells is their capacity to form stable aggregates with T cells in an antigen independent manner (Inaba and Steinman, 1986; Inaba *et al.*, 1986; Inaba *et al.*, 1989). This is irrespective of a fit between the antigen specific T cell receptor on the T cell and the MHC class II/peptide on the dendritic cell. This is thought to enhance the probability of the dendritic cell encountering the specific T cell reactive for antigen/MHC II complex from large populations of non-specific T cells. The molecular basis for this aggregation is unknown but may involve some of the surface markers described below.

Recent research over the last few years has convincingly demonstrated that a resting T cell requires 2 signals for induction of cytokine gene expression and cellular proliferation (reviewed by Schwarz, 1990 and Jenkins, 1992). The first signal is provided by the ligation of the TCR/CD3 with the antigen/MHC complex described above, and this confers the antigen specificity of the T cell. A second 'costimulatory signal' is however required to activate the T cell. These costimulatory signals are provided by the interaction of other surface molecules with their cognate ligands and are described below.

### *iv). Modulation of LC phenotype and function during migration*

The mechanism of LC function *in vivo* is not fully understood although it appears to be dependent upon granulocyte macrophage-colony stimulating factor (GM-CSF) (Heufler *et al.*, 1987, Witmer-Pack *et al.*, 1988), which will be discussed in more detail

below. During this maturation process in culture, a number of surface markers have been found to be up or down regulated which could be related to the migration and enhanced function of the LC *in vivo*.

***E-cadherin***

Cadherins are expressed in the normal, uninflamed epidermis (Nose *et al.*, 1986), and mediate cell to cell adhesion in the tissues by binding to identical cadherins on adjacent cells (Takeichi, 1990 and 1991). A recent study by Tang *et al.*, (1993) demonstrated the expression of cadherins by freshly isolated LC and, *in vitro* these LC adhered to keratinocytes through the molecule E-cadherin. Following LC culture the expression of E-cadherin was down-regulated which was associated with a decrease in the affinity of the LC for the keratinocytes. It was proposed that *in vivo* under normal resting conditions LC persist in the epidermis through the interaction of the E-cadherin molecule with keratinocytes. Following antigenic challenge, a change in the local environment, possibly mediated by keratinocyte cytokine production (discussed below) results in the down regulation of E-cadherin permitting the LC to leave the epidermis and migrate to the local lymph node.

***Intracellular adhesion molecule-1 (ICAM-1) and B7***

ICAM-1 has been shown to be important in the presentation of antigen in association with MHC class II (Dang *et al.*, 1990) by inducing important costimulatory signals through the lymphocyte function associated antigen 1 (LFA-1) of T cells (Seventer *et al.*, 1990). During LC culture, ICAM-1 expression is upregulated indicating its role in the enhanced function of the cultured LC (Tang and Udey, 1991, 1992; Simon *et al.*, 1991). There is also evidence to suggest that this ICAM-I/LFA-I interaction may be involved in the antigen independent T cell clustering described previously (Lorenz *et al.*, 1993, Makegoba *et al.*, 1989).

ICAM-1 is expressed by other cells that do not always function as antigen presenting cells and it was recently shown that the presence of an additional molecule, B7 is required for the functional maturation of the LC (Symington *et al.*, 1993). The B7/BB-

1 molecule is a member of the Ig supergene family (Yokochi *et al.*, 1982; Freeman, 1989) expressed by B cells and other antigen presenting cells. The B7 molecule binds to the CD28 and CTLA4 molecules expressed by 95% of the resting CD4 T cells and is thought to mediate the co-stimulation of $CD4^+$ T cells by regulating cytokine production (Freedman *et al.*, 1991; Razi-Wolf, 1992). Symington *et al.*, (1993) showed that cultured but not fresh LC express the molecule B7, and LC B7 co-stimulated the proliferation of resting $CD4^+$ T cells. A recent study by Larsen *et al.*, (1994) has shown that GM-CSF acts to upregulate the expression of the B7 molecules by dendritic cells, confirming the importance of GM-CSF for dendritic cell maturation.

### *v). Summary of LC function*

Epidermal LC are highly specialised to initiate primary immune responses and the antigen presenting pathway that has been described, i.e the processing of antigen in the periphery and its presentation to dendritic cells in the draining lymph. A secondary response is much more rapid and is unlikely to go through this whole sequence of events, implicating a role for the LC in presenting antigen locally to the primed antigen specific T cells present in the skin. *In vitro* studies have demonstrated the ability of freshly isolated LC to present exogenous protein antigens to peptide specific and primed T cells (Pure *et al.*, 1990, Streilein *et al.*, 1990). However the role of the LC in a secondary response has not yet been defined.

An experiment carried out by Yoshikawa *et al.*, (1992) showed that the depletion of LC from the epidermis of ultra violet B (UVB) treated skin does not inhibit the expression of pre-exisiting contact hypersensitivity, thereby implicating other antigen presenting cells of the dermis in the expression of cutaneous immunity, discussed below.

### *vi). Dermal dendritic cells*

The epidermal LC was identified as the major antigen presenting cell in the original formulation of SALT, where the dermis was thought to have only a passive role as the route by which cells entered or exited the skin. However there is increasing evidence

to suggest that the dendritic cells of the dermis play a part in the induction of immunity. Dermal dendritic cells or dendrocytes were first described by Headington (1986) as a population of bone marrow cells distinct from LC. They lack the morphological and phenotypical characteristics of LC, but share some characteristics with cells common to the mononuclear phagocyte system. Sontheimer (1989) and Cerio *et al.*, (1989) described a population of MHC class II$^+$ dendritic cells in human skin that resembled macrophages more than LC and were located within the papillary dermis and around dermal vessels. These cells expressed a surface marker factor XIIIa and the authors concluded that these factor XIIIa$^+$ dermal dendritic cells represented a population of cells separate from LC, that resemble macrophages and may have the potential for presenting antigen. Much more recent studies (from 1991 onwards) have implicated dermal dendritic cells as having a crucial role in the development of cutaneous immunity and these will be discussed in later chapters.

### *vii). The origin of LC/dendritic cells*

Dendritic cells originate in the bone marrow from as yet uncharacterised haemopoietic precursor cells and their development is poorly understood. There is some controversy surrounding the relationship of the dendritic cell with the monocyte-macrophage lineage, with the suggestion that both sets of cells arise from a common precursor. Peters *et al.*, (1991) have produced evidence supporting the concept that monocytes/macrophages represent a reservoir of pluripotent cells that differentiate into dendritic cells *in situ*. However Steinman (1983) supports the view that there is a distinct precursor cell within the bone marrow that is pre-committed to development into a dendritic cell rather than a monocyte or macrophage. Thus a common precursor in the bone marrow differentiates into neutrophils, eosinophils, basophils or monocytes. Once these cells emerge into the blood no further interconversion occurs. These separate pathways of development can be recreated in bone marrow culture using specific cytokines such as granulocyte-colony stimulating factor for neutrophils and macrophage colony-stimulating factor for macrophages (Sieff, 1987; Clark and Kamen, 1987; Metcalf, 1985). This would support the case for a separate lineage for the development of dendritic cells. It is proposed that precursor cells migrate to the

peripheral tissues such as the epidermis and reside there in an immature state as LC and it is this stage of development that is poorly understood (Breathnach, 1991). These immature cells, probably on antigenic stimulus, resume their migratory function and accumulate in the draining lymph node where they arrive as mature dendritic cells. It is believed that dendritic cells do not recirculate as they are not observed in the efferent lymph, and the lymphoid organs are the terminal stage for dendritic cells (Romani and Schuler, 1992).

Currently there is little information concerning the regulation of the maturation process or the relationship of the LC with the dermal dendritic cells. The maturational changes which occur during the culture of LC are mediated by granulocyte macrophage-colony stimulating factor (GM-CSF) and may be augmented further by interleukin-1 (IL-I) (Heufler *et al.,* 1987). In addition, GM-CSF and also tumour necrosis factor-$\alpha$ (TNF$\alpha$) (Koch *et al.,*1990) sustain LC viability *in vitro*. GM-CSF, IL-1 and TNF$\alpha$ are all products of keratinocytes. Keratinocytes represent the bulk of the epidermis and are in close contact with LC, it is therefore possible that these cytokines induce functional and phenotypical maturation of LC comparable to that observed in culture, as they migrate to the lymph node.

## 1.2.2. Keratinocytes

### *i). Cytokine production*

The keratinocyte, which constitutes the growing component of the skin, has generated considerable interest over the last ten years due to its ability to influence immunological reactions in the skin. *In vivo,* keratinocytes have been demonstrated to produce a broad range of cytokines that can modulate LC function and induce inflammation (Luger *et al.,*1988). Cytokines represent a heterogeneous group of polypeptides that mediate intercellular communications both within and between immune and non-immune cells. The cytokines produced by human and murine keratinocytes are listed in Table 1.2.

**Table 1.2** *Cytokine production by human and murine keratinocytes*

| Cytokine | Human | Murine |
|---|---|---|
| Interleukin-1 | | |
| IL-1α | + | + |
| IL-1β | + | + |
| Interleukin 6 | + | + |
| Colony stimulating factor | | |
| IL-3 | (+) | + |
| GM-CSF | + | + |
| G-CSF | + | + |
| M-CSF | (+) | + |
| Interferons | | |
| IFN-α | + | + |
| IFN-β | + | + |
| IFN-γ | ? | ? |
| Tumor necrosis factor | | |
| TNFα | + | + |
| TNFβ | ? | + |
| Transforming growth factor | | |
| TGFα | + | + |
| TGFβ | + | + |
| Platelet derived growth factor | + | (+) |
| Fibroblast growth factor | + | (+) |
| Interleukin-10 | ? | + |

Note: + = known to be produced; (+) = presumably produced, ? = unknown.

Adapted from Luger and Schwarz (1991).

As keratinocytes represent the major cell type within the epidermis and because microenvironments play a major role in dictating cellular functions, keratinocytes are now considered to be the major determinant of the intraepidermal space. Keratinocytes thus set the tone for the activities of LC and other cells within the skin that come under their influence. Under normal physiological conditions (in resting skin) cytokine production by keratinocytes is at a baseline level maintaining the immune homeostasis of the local environment. The pattern and character of cytokine production by keratinocytes is altered when the epidermis is perturbed either by physical/chemical damage or by pathogens. This alteration activates LC thereby initiating immune responses. There is evidence to suggest that keratinocytes may be responsible for the stimulus of LC migration. TNF-$\alpha$ injected intradermally results in a rapid reduction in the density of LC and a rapid and concentration dependent accumulation of dendritic cells in the local lymph node (Cumbertatch and Kimber, 1992). Kock *et al.,* (1990) have demonstrated the ability of keratinocytes to produce TNF-$\alpha$. Exposure of keratinocytes *in vitro* to UVB induces keratinocyte production of TNF-$\alpha$ and causes a reduction in LC density (Koch *et al.,* 1990) thereby supporting the role of the keratinocyte in LC migration.

### *ii). T cell interaction with keratinocytes*

In addition to the production of cytokines, keratinocytes may function as antigen presenting cells in the skin resulting in a more efficient initiation of the immune response. Keratinocytes can be induced in the presence of IFN$\gamma$ to express ICAM-1 and MHC class II molecules (Dustin *et al.,*1988, Volc-platzer, 1985). In vivo, MHC class II and ICAM-1 expressing keratinocytes have been found in skin diseases characterised by T cell infiltration (Volc-Platzer *et al.,*1988, Wantzin *et al.,*1988). As MHC class II and ICAM-1 are involved in antigen presentation with T cells, keratinocytes in the presence of cytokines may play an essential role in cutaneous immune reponses (Londei *et al.,*1984, Dustin *et al.,*1988).

The expression of MHC class II by keratinocytes following cytokine stimulation *in vivo* in the presence of costimulatory signals could enable these cells to initiate

immune responses in the absence of professional antigen presenting cells. Nickoloff (1993a, 1993b) showed that keratinocytes could provide costimulatory signals to T cells stimulated with bacteria derived antigens (known as superantigens) resulting in T cell proliferation and cytokine production. This costimulatory signal did not appear to occur through the B7/CD28 interaction that was described previously for professional antigen presenting cells. Keratinocytes do express a member of the B7 family BB-1 (or B7-3), but it is unclear if CD28-BB-1 provides T cell costimulation and it is thought that the keratinocytes deliver their costimulatory signals through unknown cell surface or soluble proteins (Nickoloff, 1993b).

Recent studies have also shown that the nature of the antigen presenting cell, professional or non-professional, may determine whether T cells become Th1 or Th2 cells (reviewed by Nickoloff and Turka, 1994). Resting T cells (Th0) have the potential to produce a variety of cytokines including IFNγ, IL-4, IL-5 and IL-10 (reviewed by Howard, 1993). The cytokine profile and/or the type of costimulatory signal provided by the two types of antigen presenting cells determines the development of the Th0 cell into Th1 or Th2 cells. The production of the cytokines IL-12 and IFNγ by the professional antigen presenting cells such as macrophages and activated B cells (Hsieh, 1993) is essential for the development of Th1 effector cells whereas the non-professional antigen presenting cells (the keratinocytes) do not produce IFNγ. Instead they synthesise IL-10 that appears to block IFNγ (Enk and Katz, 1992) and this results in the generation of Th2 cells.

Thus the cells of the epidermis have the ability to regulate the nature of the immune response to local antigens and the disruption of this cytokine balance may be important in the development of autoimmune states such as psoriasis.

### 1.2.3. Epidermotropic lymphocytes

A large number of experiments support the existence of skin-seeking lymphocytes. These cells represent a subset of recirculating T cells that preferentially migrate into the skin and reside there for an undefined period of time (homing T cells - Streilein *et al.,* 1978). T lymphocytes are absolutely critical to cutaneous immunity, acting in conjunction with antigen presenting cells to produce a specific immune response that acts both locally and systemically. Dermal vessels promote T cell immigration by the methods described below and the T cells themselves are postulated to possess specific markers that enable this process to occur. Picker *et al.,* (1990) recently identified a cell surface marker (HECA-452) that is expressed on a subset of T cells in the blood and on the majority of T cells in the skin but is rarely found on T cells in non-cutaneous sites of the body, implicating this marker as a candidate marker for skin-seeking T cells.

Ninety percent of the T cells present within the skin were found to be located in the dermis, and in contrast to their circulating counterparts, the majority of these dermal T cells were found to be activated (Bos *et al.,* 1987). There was an approximately even distribution of $CD4^+$ (helper/memory) T cells and $CD8^+$ (suppressor/cytotoxic) T cells. The majority of the T cells within the skin appeared to be of the memory cell type with naive T cells being relatively rare (< 5%) (Bos *et al.,* 1987, 1989).

Epidermotropism of T cells is prominent in many dermatological diseases ranging from rare cutaneous lymphomas (Stynavsui *et al.,* 1985, Ruiter *et al.,* 1982, Poppema *et al.,* 1983) to common benign inflammatory diseases such as eczema and psoriasis (Bjerke, 1982; Bos *et al.,* 1982; Baker *et al.,* 1984). In diseased skin it is probable that a proportion of the infiltrating T lymphocytes are antigen specific and have become activated within the skin's microenvironment.

## 1.2.4. Skin vasculature and lymphatic drainage

The S.I.S is connected to the remainder of the body's immune system by the blood and lymphatic vasculature. It was proposed early on that there is a specialisation of dermal microvessels that promote the immigration of skin-seeking lymphocytes from the blood. It has now been firmly established that the post-capillary venules are responsible for directing the migration of lymphocytes and other blood borne cells into the extravascular space (Chin *et al*, 1988). Other studies have identified cell adhesion molecules such as endothelial lymphocyte adhesion molecule -1 (ELAM-1) that are expressed by stimulated and not resting endothelial cells (Albelda and Buck, 1990). These molecules are thought to act as receptors for coreceptors expressed by leukocytes, including lymphocytes that promote migration of T cells and other cell types into the skin. *In vivo* studies demonstrate that these cell adhesion molecules are found primarily in pathological conditions and it is not known whether they are involved in promoting T cell immigration in 'resting' skin.

The skin lymphatics are essential in the generation of cutaneous immune responses. Very early studies of skin transplantation (Barker and Billingham, 1968) and contact sensitisation (Frey and Wenk, 1957) showed that the afferent lymphatics had to be intact in order to obtain immunity. The induction of a primary immune response to an antigen is dependent upon the transport of antigen to the local lymph node where the antigen can be presented to a naive T cell. The strategic location of the lymph nodes ensures that a protected site exists where naive T cells can interact productively with antigen bearing LC that have arrived via the lymph.

## 1.2.5. Other cellular components of S.I.S.

### *i). γδ-type T cells*

Another constituent of the S.I.S was identified in the murine epidermis as a bone marrow derived population of dendritic cells distinct from LC that express the Thy-1 alloantigen. They are now called dendritic epidermal T cells (DETC) (Steiner, 1988). These cells express unusually homogenous CD-2-associated TCR of γ/δ type and are

preferentially located within the epidermis. They are thought to provide a more primitive immunological defence system designed to recognise antigens in the context of non polymorphic restricting elements resembling, but distinct from, the classical class I or II molecules such as CD1 (Bucy *et al.,* 1989).

The failure to demonstrate a dense network of $CD3^+$ $\gamma/\delta^+$ cells in human epidermis led to the conclusion that a human analogue for murine DETC does not exist (Bucy *et al.,* 1989; Cooper *et al.,* 1985). However recent studies have defined a minor subset of $\gamma/\delta^+$ T cells within normal skin (Bos *et al.,* 1990). These $\gamma/\delta^+$ T cells exhibit a greater tendency to localise in the epidermis compared to the $\alpha/\beta$ CD4 and $CD8^+$ T cells. They have functional properties similar to conventional T cells (Raulet, 1989) and it is not known if they represent a human equivalent of murine DETC.

### *ii). Macrophages, mast cells and neutrophils*

Macrophages, mast cells and neutrophils are all found in low numbers within normal skin. However they can be found in large numbers in the inflammatory infiltrates observed in several cutaneous immune reactions. Macrophages serve functions related to both non-specific or natural immune reactions and specific defence systems, and have a role in the resistance to viral infections (reviewed by Unanue and Allen, 1987). The mast cells of the skin play an important role in immediate hypersensitivity reactions and non immune inflammation (Metcalf *et al.,* 1981; Lee, 1985) by regulating vasculature and T cell traffic.

## 1.2.6. The role of cytokines in viral infections

Both IFN$\gamma$ and TNF$\alpha$ exhibit potent anti-viral activities in vitro. IFN induces an anti-viral state in uninfected cells (Staeheli, 1990) and prevents viral replication and the subsequent spread to neighbouring cells. TNF$\alpha$ also acts by inhibiting viral replication (Feduchi and Carrasco, 1991) inducing an anti-viral state in the cells (Wong and Goeddel, 1986), the process of which is enhanced by the synergistic actions of IFN$\alpha$ and IFN$\beta$ (Feduchi and Carrasco, 1991; Wong and Goeddel, 1986, Mestan *et al.,*1988; Reis, 1989).

In order to determine the anti-viral role for these cytokines in vivo, Ramsay and colleagues (1993) carried out a series of experiments using recombinant vaccinia viruses (rvv) constructed to encode different murine cytokine genes, and injected them into athymic, nude mice. These mice lack mature functional T cells and die from disseminated infections with the wild type vaccinia virus. However it was found that the local expression of specific cytokines at the site of infection could control vaccinia virus replication. Infection of the mice with rvv encoding IFNγ (Kohonen-Corish *et al.*, 1990) or TNFα (Sambhi *et al.*, 1991) led to the direct clearance of the virus *in vivo*, without any evidence of enhanced host reactivity. It was demonstrated that $CD8^+$ T cells are the major producers of IFNγ (Ruby *et al.* 1994) in a vaccinia virus infection. Thus it has been been proposed that the major function of $CD8^+$ T cells following class I restricted recognition of virus infected targets is to focus anti-viral cytokines IFNγ and TNFα at the sites of viral replication (Ramsay *et al.*, 1993).

Other cytokines may have a role in the induction of local inflammatory responses to viral infections and these will be discussed in a later chapter.

## 1.3. THE CUTANEOUS RESPONSE TO ORF VIRUS INFECTION

From early on orf virus was recognised to be highly epitheliotropic, thereby inducing "tissue" immunity (Aynaud, 1923; Glover, 1928; Boughton and Hardy, 1935; Darbyshire, 1961). Some of these early studies described an influx of inflammatory cells consisting of mononuclear and polymorphonuclear cells into the dermis at the site of infection (Glover, 1928; Aynaud, 1923; Wheeler and Cawley, 1956; Abdussalam, 1957). Most of the subsequent studies were however concerned with the humoral response described previously. Failure to detect protective humoral and systemic cellular responses has led to a number of recent studies of the cutaneous responses to orf virus infection.

Techniques exist for the chronic collection of both afferent and efferent lymph in sheep (Lascelles and Morris, 1961). These techniques were employed together with a

detailed immunohistological analysis of the orf lesion to study the local immune response to orf virus. Most of the studies so far, have described the response of previously exposed sheep to a secondary infection with orf virus and the results are summarised below.

### 1.3.1. The polymorphonuclear neutrophil response

Following scarification and infection with orf virus there is a biphasic influx of polymorphonuclear cells, predominantly neutrophils into the infected skin (Jenkinson *et al.,* 1990a). The initial inflammatory response occurs within the first 24 h, well before the detection of viral antigen (the eclipse phase, Osman, 1976) and is the result of tissue damage caused by scarification. The application of the viral inoculum appears to enhance the initial inflammation in comparison with uninfected scarified skin. It is not known if this is as a result of a generalised increase in the antigenic load, or the presence of specific viral proteins. This initial influx of neutrophils into the dermis is reflected by a transient increase in the number of neutrophils detectable in the afferent lymph (Yirrell *et al.,* 1991a). Neutrophils do not appear in significant numbers in the efferent lymph and the general flow of cellular traffic in the efferent lymph is reduced transiently in the early hours following infection due to the entrapment of cells within the lymph node, a phenomenon known as "lymph node shut down".

The second phase of inflammation is observed 3-4 days post-infection and results from a reaction to the viral replication within the epidermis. At this stage viral antigen is detectable within the regenerating epidermal keratinocytes. The viral cytopathic effect is characterised by ballooning and degeneration of the infected cells. An extensive influx of neutrophils into the underlying dermis and infiltrating the degenerated ballooning cells of the epidermis is observed. Subsequently the whole degenerated mass and accumulated neutrophils are shed in the developing scab. In contrast to the first wave of neutrophil infiltration, the second wave is not reflected by the appearance of neutrophils in the afferent lymph (Yirrell *et al.,* 1991a). It is proposed therefore that there is either a stronger chemotactic signal holding the neutrophils within the lesion or they are physically entrapped within the dense mass of

cells. In efferent lymph there is a rapid recruitment phase of blood lymphocytes at this time resulting in an increased number of lymphocytes in the lymph node. This is an established response to antigen challenge.

The function of the neutrophil in the cutaneous response to orf virus is not clear. Neutrophils are typically associated with tissue damage and the clearance of bacterial infections. However neutrophils have been implicated in the clearance of some viral infections (Rouse, 1981) possibly due to the production of an interferon-like anti-viral protein termed polyferon (Rouse *et al.,* 1980; Ohmann *et al.,* 1989).

### 1.3.2. The dendritic cell response

The importance of LC/dendritic cells in the induction of cutaneous immunity was discussed in detail above. The epidermotropism of orf virus and the generation of a cutaneous inflammatory response might indicate a crucial role for LC/dendritic cells in the control of orf virus infections. Jenkinson *et al.,* (1991) carried out a study of the ovine dendritic cell response to orf virus infection. Within 'normal' ovine skin, two populations of MHC class $II^+$ dendritic cells were described, neither of which were reported to stain with the classical LC marker ATPase. The MHC class $II^+$ dendritic cells of the epidermis were found to contain Birbeck granules and stain with the enzyme marker acetylcholine-esterase (AchE) (Pearse, 1980). These cells were thus classified as ovine LC. A second population of MHC class $II^+$ dendritic cells within the dermis were negative for AchE. Following scarification and infection with orf virus a network of MHC class $II^+$ dendritic cells formed in the dermis. These cells appeared to accumulate initially as a response to scarification directly beneath the damaged epidermis. As the infection progressed this network increased, a phenomenon that was not observed in the scarified, non-infected skin. The dense accumulation of MHC class $II^+$ dendritic cells did not appear to be of LC origin as they were $AchE^-$. $AchE^+$ dendritic cells were however observed 3-4 days post-infection in the afferent lymph but only at a very low frequency compared to $AchE^-$ dendritic cells. Their appearance in the lymph correlated with the detection of viral antigen, an increased output of $AchE^-$ dendritic cells and the ability of cells isolated from the local lymph node to respond *in vitro* to orf viral antigens. Yirrell *et*

*al.,* (1994) have proposed that the $AchE^+$ cells have a role in antigen carriage to the local draining lymph node.

It is not known if AchE is expressed constitutively by ovine LC or whether it is a marker of activation/differentiation, but the $AchE^-$ network of MHC Class $II^+$ dendritic cells which gather at the orf lesion indicates that the cells share the phenotype of the dermal dendritic cells of normal ovine skin. The origin and kinetics of the MHC Class $II^+$ dendritic cell accumulation are not known. They are postulated to provide a framework for epidermal repair and immune reactions. It is thought that these dendritic cells are crucial to the control of orf virus infection, serving as a physical barrier preventing the downward spread of the virus and possibly functioning as antigen presenting cells interacting locally with T cells (Jenkinson *et al.,* 1991).

### 1.3.3. T cell and B cell responses

T lymphocytes are critical to the generation of the cutaneous immune response and also have a crucial role in the B cell production of antibodies. A study carried out by Jenkinson *et al.,* (1992) demonstrated the presence of B and T cells in the cutaneous inflammatory response to orf virus. During the initial inflammatory response to scarification and infection, T lymphocytes were observed to gather at the site of the injury. The dermal T cell number remained high and continued to increase, peaking at 72 h post-infection. The $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells were all found to be involved. The γδ T cells appeared to have a prominent role within the epidermis and pustules. The role of γδ T cells remains undefined but their frequent association with ruminant epithelial surfaces has implicated them in an immunosurveillance role (Hein and McKay, 1991). In the lymph, the majority of the afferent lymphocytes were demonstrated to have the memory phenotype in contrast to the efferent lymphocytes which are almost exclusively naive (Yirrell *et al.,* 1991b).

B cells were also shown to accumulate in response to orf virus infection, permeating the dense accumulation of MHC Class $II^+$ dendritic cells and penetrating into the infected epidermis. B cells were, however, most frequently observed in association with dermal blood vessels. It is proposed that these B cells, presumably stimulated to

produce anti-orf antibody, migrate directly into the blood, where increased levels of antibody can be detected (Yirrell *et al.,* 1989). This is supported by the absence of an increase in antibody or antibody producing cells in the afferent lymph draining the lesion (Yirrell *et al.,* 1991a). Antibody producing cells are however detected within the efferent lymph and it was determined that the major effort of the lymph node draining the orf lesion may be the production of orf virus specific antibody (McKeever *et al.,* 1987).

### 1.3.4. Comparative aspects of orf virus infection with other viral infections of the skin

The viruses vaccinia, herpes simplex virus (HSV) and human papilloma viruses (HPV) are all, like orf virus, highly epitheliotropic replicating in the epidermal keratinocytes of the skin. Cutaneous cellular immunity is proposed to be critical in the control of each of these infections.

Vaccinia virus, like orf virus, is a member of the poxvirus family and was used to vaccinate against smallpox virus infection in humans. The importance of cellular immunity in the control of vaccinia virus infections is highlighted by the progressive spread of infection in infants with defects in T cell immunity. The infection progresses through stages of papule, vesicle and pustule similar to that of orf virus infections, but when the scab that is formed is shed, it leaves a scar. The lesion is characterised by epidermal degeneration and vacuolation plus cellular proliferation (Downie, 1939). An influx of inflammatory cells including macrophages and lymphocytes is observed separating the lesion from the dermis (Buller, 1991). Systemic cellular immunity is detectable and the humoral response generated to vaccinia is thought to prevent the dissemination of the infection and to protect the host from re-infection. Locally, cell-mediated cytolytic killing of infected cells is thought to be one of the main responses in experimental animals, although cytotoxic T cells are not detected in the peripheral blood of vaccinated volunteers (Graham *et al.,*1991). Increased numbers of LC are observed at the site of inoculation in mice (Sprecher and Becker, 1989) and in vaccinated children the LC at the site of inoculation were found to contain non-replicating viral particles with an associated increase in LC acidic organelles (Nagoa *et*

*al.*, 1976). Thus LC are involved in the control of infection. More recent murine studies have implicated the Ly-1$^+$ DETC as the most important effector mechanisms in early defence against vaccinia virus infection (Ikeda *et al.*, 1991). These cells are thought to act as natural killer cells within the epidermis (Koning *et al.*, 1987).

HSV lesions differ considerably from vaccinia and orf virus lesions since they show no epidermal proliferation. During a primary infection with HSV, the virus gains entry to the local sensory nerves and establishes a latent infection within the neurones. The local cutaneous immune system appears to maintain the virus in a latent mode, but occasionally the virus reactivates causing a recrudescent lesion (reviewed by Stevens, 1989). The importance of LC in the control of HSV was demonstrated by a murine experiment whereby the density of LC at the site of inoculation was found to affect the severity of the infection (Sprecher and Becker, 1989). LC have also been shown to take up HSV (Sprecher and Becker, 1986) and act as antigen presenting cells in lymphoproliferation assays (Yasumoto *et al.*, 1986). Other cells are involved in the local inflammatory response to HSV; macrophages are thought to be important local effector cells (Kohl, 1985) and both CD4$^+$ and CD8$^+$ T cells are present. Cytotoxic T cells and T helper/delayed hypersensitivity have both been demonstrated to be essential for effective clearance of HSV from skin lesions (Wildy and Gell, 1985).

HPV infections have proved more difficult to study due to the lack of an experimental model. The importance of the immune response is however emphasised by the extensive and persistent warts seen in individuals that are immunocompromised, particularly those with depressed cell mediated immunity. Some types of HPV cause highly proliferative lesions that can persist for several months/years and, although an inflammatory infiltrate of T cells and mononulear phagocytes has been demonstrated in regressing warts (Iwatsuki *et al.*, 1986) it is not commonly observed. It is postulated therefore, that HPV may cause an alteration in the local immune response which will be described in more detail below.

## 1.4. VIRAL MECHANISMS OF IMMUNE EVASION

The immunity induced by primary infections to all four viruses; orf, vaccinia, HSV and HPV is incomplete despite high levels of circulating antibody. HSV, HPV and vaccinia virus all have the ability to alter or evade the local cutaneous response and this may be responsible for the incomplete immunity that is observed.

A direct method of immune evasion is employed by HSV. The virus establishes a latent infection in the sensory nervous system and local cutaneous immunity maintains latency. In a recrudescent infection, the inflammatory response initiated by the LC is delayed until 2 days after virus can be detected in the skin, indicating that the virus may also have other methods of evading the host response.

Studies of the lesions caused by HPV have demonstrated a reduction in LC numbers (Tay *et al.*, 1987), changes in MHC class II expression (Hughes *et al.*, 1988) and a decrease in T cell numbers with no detectable T cell activation (Tay *et al.*, 1987), suggesting that HPV may induce a local immunodefficiency by as yet unknown mechanisms thereby resulting in the development of a persistent infection.

Vaccinia virus appears to be the most adept of the three viruses at interfering with the host immune response. The extensive characterisation of the vaccina virus genome has recently identified numerous genes that contribute to viral virulence. These genes interfere with a number of different components of the host defence system and have been extensively reviewed by Smith (1993). The mechanisms include interference with the complement cascade and cytotoxic T cell responses-indicated as having an important role in combatting vaccina virus infections, and general interference with inflammatory processes though the production of serine proteinase inhibitors (serpins). The importance of the cytokines, IFN  and IL-1, in the control of vaccinia virus is emphasised by the multiple mechanisms that the virus has acquired to evade them. IFN produced by cells in response to viral infection acts by conferring an antiviral state on the cell thereby limiting viral replication and the spread of the infection (Samuel *et al.*, 1991). It has been recognised for some time that vaccinia

virus is resistant to IFN and can even rescue other sensitive viruses (Thacore and Younger, 1973a and b). One of the ways the virus confers this resistance is by preventing the IFN induction of the anti-viral state by expressing two gene products K3L (Davies *et al.*, 1992) and E3L (Chang *et al.*, 1992) that interfere with the cellular pathways of translational control. Vaccinia virus also expresses a serpin which inhibits the interconversion of the IL-1β precursor form into the secreted and active form (Ray *et al.*, 1992). In addition to these mechanisms vaccinia has recently been demonstrated to possess genes encoding soluble receptor homologues for IFNγ and IL-1β (Smith, 1993).

These soluble receptor homologues have added to the increasing family of immune evasion proteins possessed by the poxviruses. The first to be identified was a receptor homologue for TNF. Computer homology scores of the Shope fibroma virus genome revealed a gene that encodes a protein with significant homology to the ligand binding domain of the human type-1 TNF receptor (TNFR-1) (Smith *et al.*, 1990). When the recombinant protein was expressed it was found to be secreted as a soluble protein that specifically binds TNFα and TNFβ with high affinity (Smith *et al.*, 1991). Myxoma virus, another orthopoxvirus, has also been identified as possessing a TNFR homologue. Vaccinia virus contains a sequence homologous to TNFR (Howard *et al.*, 1991) but the identified open reading frame is interrupted by a frame shift and a termination codon that suggests that an intact receptor protein is unlikely.

Virally encoded proteins have been shown to help the virus evade the host immune surveillance mechanisms, participate in the regulation of deleterious cytokine production and interfere intracellularly with host derived anti-viral mechanisms. Upton *et al.*, (1991) predict the existence in the most pathogenic poxviruses of multiple cytokine receptors, termed viroreceptors, each targeted for a different pathway dependent upon lymphokine-receptor interaction.

## 1.5. AIMS OF THESIS

The local cutaneous immune system is clearly important in the control of viral infections of the skin but little is known about the interactions of the specific virus with the cells involved in generating a protective immune response to the infection. Orf virus infection of sheep provides an opportunity to study local *in vivo* immunity to a skin tropic virus in its natural host where the skin lesion is localised and visible, and and where there is no systemic disease.

The local cutaneous response to orf virus infection involves a number of different cell types. Central to this response is a network of MHC class $II^+$ dendritic cells that are postulated to form a framework for epidermal repair and immunological reactions. This thesis is designed to further knowledge of the local cellular response to orf virus by characterising in detail the dendritic cell network of both the primary and secondary orf lesions. The relationship of these MHC class $II^+$ dendritic cells with the dendritic cells normally resident within the skin, the kinetics of the response and how, or from where, the network of dendritic cells originates will be examined (see chapter 3). In addition, pure cultures of ovine keratinocytes will be infected with orf virus *in vitro* to determine the kinetics of viral replication and CPE (see chapter 4).This *in vitro* model of infection will then be used to investigate cytokine production by ovine keratinocytes infected with orf virus, to determine the ability of the virus to induce or suppress a range of cytokines which may play an important role in the resolution of an orf virus infection *in vivo* (see chapter 5).

# CHAPTER 2

## MATERIALS AND METHODS

## 2.1. ORF VIRUS INFECTION OF SHEEP

### 2.1.1. Sheep

Female Suffolk cross sheep, reared at the Moredun Research Institute, were used in all the experiments.

**Group 1. Primary orf infection.**

Four specific pathogen free (SPF) lambs were isolated from birth and kept in an environment free from orf virus before and during the experiment. When two months old, all four animals were given a primary infection with orf virus following the method described below.

**Group 2. Secondary orf infection.**

Four conventionally reared sheep, aged between 12 and 14 months old, were used. Blood samples were taken from the animals prior to the start of the experiment and analysis by ELISA (described below) revealed positive titres to orf virus antigen which indicated previous exposure to orf virus in the field. The animals were challenged experimentally with orf virus, as outlined below.

**Group 3. Control scarification.**

Four conventionally reared control sheep, aged between 12 and 14 months old, were scarified experimentally but not challenged with orf virus to act as controls.

### 2.1.2. Viral inoculum and inoculation procedure

The virus used in the experiments was Scabby Mouth, a vaccine produced by the Commonwealth Serum Laboratory of Australia consisting of live tissue culture propagated orf virus which has retained virulence. All 12 animals were scarified on the inner thigh with a single stroke using a 16 gauge needle. To groups 1 and 2, 0.1ml of the vaccine ($10^7$ tissue culture infectious dose ($TCID_{50}$)/ml) was applied topically to the treated skin. To the abrasions of group 3, 0.1ml of phosphate buffered saline

(PBS) was applied. Skin samples (6mm in diameter) were taken by punch biopsy from all 12 animals across the scar lines at 0h, 48h, 96h and 170h after treatment. Extra biopsies were collected from the primary infected animals at days 9, 12, 16, 22 and 30 for qualitative observations. The biopsies were immediately placed into formaldehyde cacodylate fixative [20mls formaldehyde, 13.7g of sucrose, 3.424g of sodium cacodylate (B.D.H, Poole, Dorset, UK) in distilled water].

### 2.1.3. ELISA for the detection of orf virus antibodies in ovine sera

Ovine sera were screened for antibodies to orf virus following the procedure of Yirrell *et al.*, (1989) and was carried out on my behalf by Janice Gilray. In brief, alternate wells of a microtitre plate was coated with either control antigen (prepared from monolayers of foetal lamb muscle cells (FLM)) or orf viral antigen (prepared from orf virus infected monolayers of FLM) in coating buffer at 4°C overnight. After washing, doubling dilutions of the test sera, from 1:100, were added in duplicate to the plate along with positive (ovine hyperimmune sera) and negative (sera from SPF lambs) controls and incubated at room temperature for 2 hours. After further washing steps, a secondary antibody, donkey anti-sheep IgG horse radish peroxidase conjugate (Sigma), was allowed to react for a similar time. O-phenylenediamine (OPD, Sigma) and 30% hydrogen peroxide were added for 3 min at room temperature before the reaction was stopped by the addition of 2.5M $H_2SO_4$. The end point titre was calculated as the highest dilution greater or equal to the mean of eight wells (four control and four orf antigen) of a 1/100 dilution of sera from SPF lambs.

### 2.1.4. Immunohistology

#### *i). Tissue Processing*

Each skin specimen was fixed for 2-3h at 4°C in the cacodylate/formaldehyde fixative (described above) and then stored for up to 18h in 0.4M sucrose at 4°C (Jenkinson *et al.*, 1991). The specimens were halved; one half was frozen in liquid nitrogen and then stored at -70°C. The other half was processed to paraffin wax by the St.Marie method (Pearse, 1980). In brief, the tissue was placed into plastic cassettes and left overnight in 70% ethanol at 4°C. The next day the tissue was processed through half-

hour changes at 4°C of; absolute alcohol (three incubations), equal parts absolute alcohol/xylene (two incubations) and then into xylene (at room temperature) before being processed under vacuum into wax. For quantitation or semi-quantitation of dendritic cells by immunohistology, 10μm thick serial sections of each of the biopsies were cut and 5 sections of at least 50μm intervals apart, mounted on the same slide pre-coated with Biobond (British Biocell, Cardiff). Extra non-serial 10μm paraffin sections from primary lesions were cut for non-quantitative assessment. Sections of fixed, frozen tissue were cut at 10μm thick in a cryostat for immunostaining with some of the antibodies (see below).

### *ii). Avidin-biotin immunoperoxidase technique*

Using an avidin/biotin immunoperoxidase technique (Vectastain Elite, Vector Laboratories Ltd, Burlinghame, USA) a set of slides from each sheep was immunostained with a panel of specific antibodies listed below.

The paraffin tissue sections were deparaffinized through xylenes and graded alcohol series and rinsed in distilled water. Both frozen and paraffin sections were washed for 20 min in PBS buffer pH 7.5 (3.8g sodium phosphate, 9g sodium chloride dissolved in 1 litre of distilled water and adjusted to pH to 7.5 with hydrochloric acid, with the addition of 0.5ml Tween 80) and blocked for 20 min with normal horse serum diluted (1:4) in high salt PBS buffer (prepared by the addition of 2g of sodium chloride to 100mls of PBS wash buffer with no Tween 80). The excess serum was blotted from the sections (without letting them dry) and the sections incubated with primary antibodies overnight at 4°C in a moist box. The slides were washed in PBS buffer for 10 min and incubated with the Vectastain Elite secondary antibody solution, biotinylated horse IgG anti-mouse immunoglobulin (2.5μl biotin conjugate and 17.5μl normal horse serum diluted in 1ml of high salt PBS buffer) for 30 min at room temperature. For the rabbit antiserum against factor XIIIa, biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) was used as the second stage reagent. After another wash any endogenous peroxidase activity in the tissue was quenched by incubation in 1% hydrogen peroxide in methanol for 15 min. The sections were

washed in PBS buffer for 10 min and subsequently incubated with Vectastain Elite ABC reagent (20µl of reagent A and 20µl of reagent B in 1ml high salt PBS buffer) for 30 min, again at room temperature. After a final wash in buffer the slides were incubated in peroxidase substrate solution - diaminobenzidine (DAB) substrate kit (Dako Ltd, High Wycombe) 1 drop of buffer, 2 drops of DAB and 1 drop of hydrogen peroxide into 2.5mls of distilled water (mixing in between each addition) until staining could be seen under the microscope (approximately 2-7 min) and the DAB was washed off the sections under running tap water. All slides were counterstained in haematoxylin and Scott's tap water (3.5g of sodium bicarbonate and 20g of magnesium sulphate dissolved in 1 litre of distilled water), dehydrated through graded alcohols and histoclear (National Diagnostics, New Jersey) and mounted in histomount (National Diagnostics).

### *iii). Double staining immunoperoxidase/alkaline phosphatase technique*

For two colour analysis, a second monoclonal was applied after the first had been coloured by DAB and was incubated for an hour at room temperature. The incubations were repeated as described previously but the second stage reagent was exchanged for an alkaline phosphatase labelled avidin-biotin complex (also supplied by Vector laboratories). The enzyme was visualised using a different coloured substrate; New Fuschin (Vector laboratories) that gave a red/violet colour. The slides were counterstained and mounted as described before.

### *iv). Antibodies*

Each antibody (in high salt PBS buffer) was titrated on ovine tissue and the optimal working dilution determined.

#### *MHC class II*

Murine monoclonal IgG 2a antibody SBU II 49.1, an anti-MHC class II antibody that recognises an epitope common to ovine DR and DQ (Puri *et al.*, 1987) was used at a dilution of 1:50. It was obtained from Dr Mal Brandon, University of Melbourne.

*SBUT 6*

Murine monoclonal antibody SBU T6 20:27, an anti-ovine, pan CD-1 specific antibody that recognises an antigen expressed on the surface of cortical thymocytes, epidermal/dermal dendritic cells and a subset of B lymphocytes (Mackay *et al.,* 1985 and Howard *et al.,* 1987). The optimal working dilution was 1:4. It was obtained from Dr Mal Brandon, University of Melbourne.

*Factor XIIIa*

Rabbit IgG anti-human coagulation factor XIIIa (Calbiochem-Novobiochem, La Jolla, USA) has recently been used to identify a subgroup of dermal dendrititc cells which coexpress MHC class II antigens in humans (Cerio *et al.,*1989). The optimal working dilution was 1:750.

*Anti-PCNA*

The monoclonal antibody to proliferating cell nuclear antigen (PCNA), clone PC-10 (Dako Ltd, High Wycombe), recognises a phase specific nuclear antigen that functions as a co-factor for DNA polymerase delta (Linden *et al.,*1992). PCNA levels are increased through the mid G1 phase of the cell cycle, remain elevated through the S phase, then rapidly decrease from G2/M to G1 (Bolton *et al.,*1992). The working dilution of the antibody was 1:200.

*Anti Orf virus*

Murine monoclonal antibody 5E2, specific for an unidentified orf virus protein, was used at a dilution of 1:200. It was obtained from Dr H.W.Reid, Moredun Research Institute, Edinburgh.

*Monocyte/Macrophage markers*

Five monoclonal antibodies that recognise antigens on the surface of monocytes/macrophages were used. The OM monoclonals were used on sections of frozen tissue as they gave unreliable results on paraffin sections.

*OM1, 2 and 3*

The murine monoclonals OM1, 2 and 3 were developed against ovine alveolar macrophages and recognise both monocytes and tissue macrophages (Pepin *et al.*, 1992). OM1 has recently been identified as an anti-ovine CD11c (Gupta *et al.*, 1993). OM2 and OM3 recognise a subpopulation of alveolar macrophages and are not specific for the monocyte-macrophage lineage. OM2 also labels vascular endothelium. The working dilution of the antibodies was 1:5. They were obtained from Dr M.Pepin, Institut National de la Recherche Agronomique, France.

*IL-A15 and IL-A24*

Antibodies IL-A15 and IL-A24 (ILRAD laboratories, Kenya) predominantly recognise monocytes and granulocytes. IL-A15 is an anti-ovine/bovine CD11b monoclonal (Splitter and Morrison, 1991). IL-A24 anti-ovine/bovine P110/75 antigen recognises a subpopulation of dendritic cells as well as neutrophils, eosinophils, macrophages and some lymphocytes (McKeever *et al.*, 1991). The optimal working dilution of both antibodies was 1:1000.

v). *Controls*

Negative controls included isotype matched murine monoclonal antibodies specific for border disease viral epitopes (obtained from Dr G. Entrican, Moredun Research Institute, Edinburgh) and rabbit serum IgG as a control for the factor XIIIa specific antibody. Monocyte-macrophage markers were tested for their activity on formaldehyde/cacodylate fixed cytospins of ovine bone marrow cells and cultured ovine alveolar macrophages (obtained from Dr G. Entrican, Moredun Research Institue, Edinburgh).

*vi). Quantification of dendritic cells*

Dendritic cells were defined by reported criteria (Steinman, 1991). In particular they were mononuclear (with round or indented nuclei) cells with prominent dendritic processes. Dendritic cells positive for MHC class II, Factor XIIIa and CD1, were

individually quantified within a total area of 1mm$^2$ of dermis on five semi-serial sections of each biopsy from normal skin and at the time-points 48h, 96h and 170h after treatment (Jenkinson *et al.*, 1991). The counts were performed on four microscope fields across the length of the section of skin. Dual staining of dendritic cells with 2 of the 3 antibodies was determined by comparison of serial sections or by the two colour technique described. Counts were not performed for cells other than dendritic cells or for the additional sections of primary orf lesions collected at timepoints beyond 170h. Comparisons between consecutive times, between treatments at the same time and for the different dendritic cell markers were made.

## 2.2. ORF VIRUS INFECTION OF KERATINOCYTES

### 2.2.1. Keratinocyte culture

#### *i). Primary culture*

The method of Weterings *et al.*, (1981) was followed in outline. Hair follicles were plucked from the muzzle of young lambs (up to 6 months old) that were restrained but not anaethetised. Prior to plucking, the muzzle area was wiped with cotton wool soaked in microgen to reduce the risk of contamination. The longer hairs were plucked using eyebrow tweezers and placed directly into culture medium. The culture medium was composed of Dulbeccos Minimum Essential Medium (MEM, ICN Flow, High Wycombe, UK) buffered with 8% sodium bicarbonate containing 0.4% phenol red, with 2% Ultroser G (Gibco BRL, Uxbridge, Middlesex, UK), 6% foetal calf serum (heat- inactivated, Gibco), 1% glutamine, (L-glutamine, Sigma) 1% non-essential amino acids, gentamicin (25μg/ml, Roussell ) and fungizone (0.5 units/ml, Squibb) and for primary cultures only, the growth factors epidermal growth factor (10ng/ml, Boehringer Mannheim, Lewes East Sussex, U.K) and hydrocortisone (0.4μg/ml, Sigma) were included. The hairs were poured into petri dishes and the bulk of the hair shafts cut off using a sterile scalpel. Each bulb was then placed on the side of 25 cm$^2$ flasks (Corning Inc, Corning, New York) and left to attach for about 5 min. Primary culture medium was then pipetted down the side of the flask free of bulbs and then the flask was gently tilted into a horizontal position so that the bulbs

were covered in medium without being disturbed. The cultures were incubated at 37°C in a humified atmosphere of 5 % $CO_2$ in air and left undisturbed for 10 days, after which the medium was routinely replaced twice a week. When the keratinocyte colony surrounding the hair bulb had reached ≥1cm (2-3 weeks incubation) the cells were ready for subculture.

### *ii). Subculture*

Monolayers of keratinocytes were rinsed twice with PBS and detached from the flasks by incubating with 1ml/flask of 0.25% trypsin/EDTA at 37°C for 10-15 min. Once the cells were detached, 2mls of culture medium (no growth factors included) were added to the flask to neutralise any remaining trypsin. The detached cells were resuspended by pipetting vigorously and then centrifuged at 300g for 5 min to pellet the cells. The pellet was resuspended in 1ml of culture medium and the single cell suspension put back into the original flask and 8mls of culture medium (no growth factors) added. A monolayer formed in the flasks after about 3-5 days, at which point the cells could be subcultured into flasks by the method already described.

### *iii). Cryopreservation*

Cells were stored in liquid nitrogen at pass 2. The cells were detached from the culture flasks as described above and after 1 wash the cells were resuspended in 1ml/flask in medium containing 10% dimethylsulphoxide (DMSO, B.D.H) and 50% foetal calf serum and transferred into a cryotube (Nunc Inc, Naperville, Illinois). The tubes were then wrapped in cotton wool and stored at -80°C and 24 hours later, the tubes were transferred to a liquid nitrogen tank, where they were stored until required. In recovery from frozen storage, the cells were thawed rapidly in a 37°C water bath and the suspension diluted in culture medium. The cells were washed and inoculated into 25 $cm^2$ flasks (1vial per flask).

iv). *Growth of keratinocytes on plastic coverslips.*

A suspension of keratinocytes diluted 1:10 in trypan blue (0.2% in PBS, Gurr) was counted in a modified Fuch's Rosenthal counting chamber (Weber) and the cells diluted in medium to give a final concentration of $1x10^5$ cells/ml. This suspension was seeded at $1x10^5$ cell/ml into 24 well tissue cluster plates containing 13mm diameter Lux Thermanox coverslips (Nunc). Foetal lamb skin cells provided by the tissue culture department at the Moredun Research Institute were also cultured on the coverslips. After 2-3 days incubation at 37°C, the cells formed a monolayer and were ready for further experimentation.

Coverslips of keratinocytes and foetal lamb skin cells were fixed with formaldehyde/cacodylate and stained for morphological observation with the Leishman's stain (eosin-methylene blue, Gurr) by immersing the slides in the neat solution for 2 min and then for 10 min with a solution of Leishman's diluted 1:2 in distilled water before rinsing with tap water and air drying. The dried cells were then mounted with DPX (B.D.H).

*v). Immunohistochemical study*

Keratinocyte cultures were incubated with a number of monclonal antibodies that will distinguish keratinocytes from other contaminating cells such as fibroblasts, in order to give a rough estimate of the purity of the established cultures. Foetal lamb skin cells were included as controls as they constitute a mixed population of cells, the majority of which are fibroblastic in nature.

*Fixation*

Monolayers of keratinocytes and foetal lamb skin cells grown on plastic coverslips as described above were used. The medium was aspirated off using a vacuum suction pump and the cells rinsed twice with PBS. The cells were then fixed with 0.5 mls/well of ice cold acetone for 10 min. The acetone was aspirated off and the coverslips rinsed with distilled water. Using forceps, the coverslips were lifted out of the wells and laid on blotting paper to air dry. The coverslips were attached to glass slides on

the non-cell side using the mountant DPX (B.D.H), and after the mountant had solidified, the slides could be immmunostained.

***Monoclonal antibodies***

Monolayers of cells were immunostained using the enhanced avidin-biotin immunoperoxidase kit (Vectastain Elite) method described above.Three antibodies were used;

Anti- Keratin, an antibody that specifically stains networks of tonofilaments within epithelial cells. The antibody was used at a dilution of 1:40 (guinea pig anti-keratin, Sigma K-4252)

Anti-Pan cytokeratin, a monclonal antibody that detects three cytokeratin proteins expressed in simple, cornifying and non-cornifying squamous and pseudostratified epithelia. The antibody was used at a dilution of 1:50 (mouse anti-cytokeratin, Sigma C-1801)

Anti-Vimentin, is immunospecific for vimentin, a cytoskeletal protein localised within fibroblasts. The antibody was used at a dilution of 1:100 (mouse anti-vimentin, Sigma V-5255).

## 2.2.2. Orf virus

### *i). Preparation of orf 11 and Scabby Mouth.*

Two tissue culture adapted strains of orf virus, orf 11 and Scabby Mouth were used in the *in vitro* work. The viral stocks were prepared on my behalf by Irene Pow and Janice Gilray. In brief, orf 11 was prepared from a scab virus isolate (McKeever, 1986) and adapted to tissue culture by passaging in different types of sheep cell; 22 passages in thyroid cells, 4 passages in foetal lamb muscle cells and 2 passages in foetal lamb skin cells. The stock virus was prepared from foetal lamb skin cells infected with the tissue culture adapted virus . Ninety-six hours post-infection, when the cytopathic effect was at a maximum, the cells were subjected to 3 cycles of freezing and thawing to release cell bound or cell-associated virions into the

supernatant. Any cells still attached to the flask were scraped into the medium with a rubber policeman. The virus-cell mixtures from the flasks were pooled and clarified at 1200g for 30 min at 4°C. The resulting supernatant was then titrated and stored as stock virus at -80°C.

The Scabby Mouth vaccine orf virus strain has been adapted previously to tissue culture and was passaged in foetal lamb skin cells twice before production of a "master" stock of virus on the third passage in foetal lamb skin cells . This master stock was then used to produce working stocks of virus by inoculating flasks of foetal lamb skin cells and collecting the virus as described above.

### *ii). Titration of the stock viruses in keratinocytes.*

Twenty-four well plates containing monolayers of keratinocytes grown on plastic coverslips as described in section 2.2.1 (iv), page 46 were used for each of the two viral preparations. The supernatant from the cultures was aspirated off and the monolayers washed twice with PBS. Ten-fold viral dilutions from $10^{-1}$ to $10^{-7}$ were prepared in keratinocyte medium from the stocks of virus and 0.1ml of each dilution added to 6 wells. The plates were incubated at 37°C for 1 hour following which the inoculum was aspirated off, the monolayers washed twice with PBS, 0.5mls of medium added and the incubation at 37°C continued. The monolayers were examined at 12h, 24h and then daily for 4 days for cytopathic effects (CPE), indicated by cell rounding, clumping or detachment of cells from the coverslip. At the end of 4 days the total proportion of positive wells (i.e the wells that showed CPE) at each dilution was summed and used to calculate the 50% tissue culture infectious dose ($TCID_{50}$) of the stock virus. At each time point, coverslips from the $10^{-1}$ wells were fixed in acetone or formaldehyde/ cacodylate for Leishman's staining and immunohistochemistry.

### *iii). Viral growth curves*

A one step growth curve was carried out to determine the various stages of viral replication for Scabby Mouth and orf 11 in keratinocyte cultures.

Fourteen 25cm$^2$ flasks of keratinocyte monolayers were used for each of the two virus preparations. The medium from the flasks was aspirated off and the monolayers washed with PBS. The cells were infected with 1ml of inoculum/flask (a multiplicity of infection of approximately 0.5) and adsorption carried out at 4°C for 1 hour. At the end of the adsorption period the monolayers were washed with PBS to remove any unadsorbed virus. Seven mls of growth medium was added to each flask which were incubated at 37°C. Two flasks for each viral inoculum were removed from the incubator at 0h, 4h, 8h, 12h, 24h, 48h, and 72h post-infection. On removal, the growth medium was collected into universal containers which were stored immediately at -70°C. The monolayers were washed with fresh growth medium and also stored immediately at -70°C with a covering of about 3mls of medium/flask. At the end of the incubation, all flasks and universals were removed from -70°C and subjected to three cycles of rapid freeze-thawing. This procedure disrupts the cells and releases any intracellular virus particles. After the final freeze-thawing step the samples were centrifuged at 1000g for 10 min to pellet any cellular debris. The virus particles obtained from the disrupted cells and supernatants were diluted in ten fold steps to $10^{-9}$ and titrated on to monlayers of keratinocytes grown in 96 well plates. At 96h the plates were read for cytopathic effect and the infectious virus titre ($TCID_{50}$) of the supernatant and the cells for both viral preparations at each time point was determined as previously described.

*iv). Detection of orf viral antigen in infected keratinocytes.*

Two methods were used for detecting expression of orf viral antigens in monolayers of infected keratinocytes at various time points post-infection.

***Immunofluorescence.***

Keratinocytes did not monolayer well on glass substrates, the best monolayers being achieved on plastic coverslips. However plastic coverslips autofluoresce under UV light and so, for immunofluorescent studies, keratinocytes were grown to as near a monolayer as possible on glass coverslips.

A suspension of keratinocytes at $1x10^5$ cells/ml was seeded into test-tubes (Greiner) containing glass coverslips. The test-tubes were held at an angle and the cells incubated at 37°C for 2-3 days until they had monolayered. The medium was aspirated off, the coverslips rinsed twice with PBS and 1.5 mls of dilutions of virus $10^{-2}$ , $10^{-3}$ and $10^{-4}$ (prepared in medium) added to the tubes which were incubated at 37°C for 1 hour to allow viral adsorption. The inoculum was then aspirated off, the cells rinsed with PBS and 1.5mls of fresh medium added to each tube and the incubation continued at 37°C. Coverslips from each dilution and the uninfected controls were fixed by the addition of ice cold acetone (as previously described) at 0h, 6h, 12h, 18h, 24h and 48h post-infection. The coverslips were then laid to dry on blotting paper and then attached to glass slides, cell-side up using the mountant DPX.

An indirect staining method was used for immunofluorescence. The coverslips were first incubated with horse serum (Gibco) diluted 1:20 in PBS at 37°C for 30 min in a humidity chamber to reduce any non-specific binding. The coverslips were then rinsed with PBS (2 x 10min). Any excess PBS was carefully dabbed off with tissue paper and the coverslips incubated for 30 min at 37°C with a 1:50 dilution of whole serum from a hyperimmune lamb D573. This lamb had been experimentally infected with orf virus, the scab collected, and at 16 and 18 weeks post-infection it was inoculated intramuscularly with autogenous scab suspended in Freund's complete adjuvant (Difco, East Molesey, Surrey, U.K). It was bled 10 days after the second inoculation and the serum stored at -20°C. After the primary antibody stage, the coverslips were rinsed with PBS (2 x 10 min), and donkey anti-sheep FITC conjugate (Scottish Antibody Production Unit, SAPU) diluted 1:40 in PBS applied for 30 min at 37°C . After a final rinse in PBS (2 x 10 min) the coverslips were mounted in glycerol and viewed under the UV microscope (Ortholux-Leitz).

***Immunoperoxidase***

Acetone-fixed monolayers of orf-infected keratinocytes grown on plastic coverslips (at the time-points described above) were incubated overnight at 4°C with the monoclonal anti-orf antibody 5E2 (ascites 1:200, Dr H.W.Reid, Moredun Research

Institute, Edinburgh) and the immunoperoxidase stain carried out with the Vectastain elite kit as described in section 2.1.4 (i), page 40.

### *v). Incorporation of $^{35}S$ amino acids by keratinocytes.*

#### *Orf virus infection of Keratinocytes*

6 x 25 $cm^2$ flasks of keratinocytes were grown to 80 % confluence in keratinocyte growth medium. Twenty-four hours prior to infection, the flasks were rinsed twice with PBS and the medium replaced with methionine and cysteine-free DMEM medium containing 2% FCS and 1 % glutamine. The medium from the flasks was then removed and the flasks rinsed with PBS. Four of the flasks were inoculated with 1 ml of orf virus (orf 11, multiplicity of infection (MOI) 0.5 to 1)and 2 control flasks received 1 ml of the methionine and cysteine-free medium containing 2% FCS. All 6 flasks were incubated at 37°C for 1 hour. After the incubation, the virus was aspirated off, the flasks rinsed with warm PBS and pulsed with $^{35}S$ amino acids as described below.

#### *Incorporation of $^{35}S$*

The $^{35}S$ work was carried out on my behalf by David Deane.
The flasks were divided into 2 sets of 3 flasks, containing 1 control flask and 2 infected flasks. Five ml of methionine and cysteine free DMEM (ICN, Flow, without FCS) was added to the uninfected flask and one of the infected flasks of each set. The other infected flask of both sets received 5 ml methionine and cysteine free medium DMEM (without FCS) containing 1μg/ml of actinomycin D. One set of flasks (Early set) was immediately pulsed for 2 hours with 100μCi of $^{35}S$-methionine (Amersham International, Little Chalfont, England). After pulsing the flasks were washed and chased for 5 hours with DMEM (without FCS) containing methionine and cysteine. The cells were scraped off into the culture medium and harvested by centrifugation at 2000rpm for 5 min. The supernatants were carefully aspirated and both the cell pellet and supernatant for each sample stored at -20°C. After 6-7 hours incubation at 37°C, the second set of flasks (Late set) were pulsed with $^{35}S$-methionine for 2 hours,



washed and chased in DMEM containing methionine but no FCS and harvested as for the Early set.

*Trichloroacetic acid (TCA) precipitation-spotting*

The frozen cell pellets were lysed in 1% Nonidet P40 in PBS and 10µl of cell lysates and supernatants were spotted onto dry, glass fibre filter disks (20mm diameter, Whatman) and left to air dry. Once dry, the filter disks were immersed in a beaker containing a 10 % solution of trichloroacetic acid (TCA) in water, and incubated on ice for 30 min. The disks were then rinsed (x 2) with fresh 10 % TCA and washed extensively (2 to 3 times) with 95 % ethanol in water. The filters were placed on aluminium foil in a hood and left to dry. Once dry, the filters were placed into scintillation vials with 10 ml of scintillant (Picofluor 15, United Technologies, Packard) and counted in a scintillation counter.

*Autoradiography*

10 µl of each sample was loaded onto a 5 %, 12 % discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and run under reducing conditions at 200V for 1 hour. The gel was then stained with approximately 10ml of 0.02 % Coomassay brilliant blue (in 30% methanol 10% acetic acid) for 15 min. The gel was then de-stained for 1-2 hours and impregnated with 10ml of Amplify scintillant (Amersham) to enhance the signal. The gel was placed onto 3mm paper (Whatman) and dried in a flat bed heater under vacuum for 1 hour. The dried gel was then autoradiographed with hyperfilm MP (Amersham) in an X-ray cassette overnight at -70°C. The gel was then developed under the appropriate light conditions, using D19 developer and Kodafix (Kodak Ltd), diluted as per the manufacturer's instructions.

## 2.3. ANALYSIS OF CYTOKINE PRODUCTION BY KERATINOCYTES

### 2.3.1. Experimental procedure

Keratinocytes were analysed for their ability to produce cytokines under normal resting conditions, in response to infection with orf virus, after UV irradiation and after stimulation with phorbol 12-myristate 13-acetate (TPA)/Calcium ionophore.

*i). Orf virus infection of keratinocytes*

25cm$^2$ flasks of keratinocytes were washed twice with PBS and 1ml of either orf 11 or Scabby Mouth virus was added to each flask at an M.O.I of approximately 0.5; keratinocyte medium was added to an equal number of control flasks. The flasks were incubated at 4°C for 1 hour on a rocker to allow adsorption of the virus. At the end of adsorption (time 0h), the flasks were rinsed with PBS, 8mls of keratinocyte medium (containing 1%, 2% or 6% FCS) added to each flask and the flasks incubated at 37°C. Control and infected flasks were removed at 2 hourly intervals from 0 hours up to 12 hours and then at 24 hours post-infection. On removal from the incubator the cell-free supernatants were collected into universal containers and stored at -20°C. The monolayers were rinsed twice with PBS and the cells lysed by the addition of 1ml/flask of guanidine isothiocyanate (G.I.T.) extraction buffer (6M guanidium isothiocyanate, 250mM sodium citrate pH 7, 0.5% sarcosyl and 100mM 2-mercaptoethanol). The cell lysate was collected and stored at -20°C for the subsequent isolation of RNA.

ii). *UV irradiation of Keratinocytes*

Monolayers of keratinocytes were grown in 18 small petri dishes (5cm in diameter, Cell Cult). Immediately prior to exposure to UV light, the medium was removed and the cells covered with the minimum volume of PBS to keep them wet. Three sources of UV light were used; UVB broad band TL12 (270-320nm, peaking at 310nm) which emits 4.8mJ/cm$^2$ in 1min, TL01 UVB narrow band (312 nm) emits 13mJ/cm$^2$ in

1min and UVA (310-400nm, peaking at 310nm) emits 60mJ/cm$^2$ in 1 min. Six experimental groups were set up as follows and during exposure to UV, the lids of the petri dishes were removed.

Two groups were exposed to a UVB broad band Phillips TL12 lamp; group 1 was given a high dose exposure of 4 min 12 seconds (20mJ/cm$^2$) and Group 2 was exposed to a low dosage 2 min 6 seconds (10mJ/cm$^2$). Groups 3 and 4 were exposed to a UVB narrow band Phillips TL01 lamp at high and low exposures (50mJ/cm$^2$, 4 min 12 seconds and 20mJ/cm$^2$, 1 min 42 seconds respectively). Group 5 was exposed to 1 minute 42 seconds of UVA light (100mJ/cm$^2$) and a control group 6 that was unexposed was included.

After irradiation the PBS was removed from the dishes and replaced with 1ml of medium. The dishes were then incubated at 37°C for 24 hours after which the morphology of the cells was noted, the supernatants collected and the cells lysed into extraction buffer as described above for subsequent cytokine analysis.

iii). *Phorbol 12-myristate 13-acetate (TPA)/calcium ionophore (Ca) stimulation of Keratinocytes*

Monolayers of keratinocytes in 25cm$^2$ flasks (Corning) were rinsed twice with PBS. 10mls of growth medium containing 5ng/ml of TPA (Sigma) and 500ng/ml of calcium ionophore (Ca) (Sigma) was added to each flask. An equal number of control flasks received 10mls of growth medium and the flasks incubated at 37°C. Control and stimulated flasks were removed at 4 hour intervals from 0 hours after stimulation up to 12 hours. The culture supernatants and cells were collected and stored for subsequent cytokine analysis as described above.

## 2.3.2. Cytokine mRNA detection:

Keratinocyte cytokine mRNA was measured using a reverse transcriptase polymerase chain reaction (RT-PCR) technique.

*i). Extraction of total cellular RNA*

The solutions containing the lysed cells were thawed and homogenised using a $20^{1/2}$ gauge needle and the pH lowered by the addition of a 1/10th volume of 2M sodium acetate (pH 4.5). An equal volume of water saturated phenol followed by 1/5th volume of chloroform/isoamyl alcohol 49:1 (water saturated) were added, mixing in between each addition.The aqueous and solvent phases were then separated by centrifugation in a microfuge at 13,000 rpm for 10 min. The upper aqueous layer was transferred into a fresh tube and an equal volume of isopropanol added. The solution was left at -20°C for 1 hour and RNA pelleted by centrifuging at 13,000 rpm for 10 min. The supernatant was removed and the pellet dissolved in 100µl of the G.I.T. extraction buffer, 100µl of isopropanol added, mixed and stored at -20°C for 1 hour. The RNA was re-pelleted by centrifuging at 13,000 rpm for 10 min, the supernatant removed and the pellet washed in 70% ethanol. Finally the RNA pellet was lyophilised in a dessicator and then dissolved in 50µl of diethylpyrocarbonate (depc) treated water (sterile, RNase-free water, produced by adding a solution of 1ml depc (Sigma) and 5 mls ethanol to 1 litre of distilled water, incubating overnight and then autoclaved to destroy the depc).

*ii). Estimation of RNA concentration*

The concentration of RNA in each of the samples was measured spectrophotometrically at 260/280nm (Beckman DV650) and the results confirmed by checking the intensity of the bands after running equal concentrations of the RNA on a denaturing 1% formaldehyde/agarose gel.

The gels were prepared by dissolving 0.4g of agarose (Gibco BRL) in 34mls of distilled water in a microwave oven. Once the agarose had cooled below 65°C, 4mls of 10x MOPS buffer [0.2M MOPS 3-(N-Morpholino) propanesulphonic acid (Sigma), 50mM sodium acetate and 10mM EDTA pH7] and 2mls of formaldehyde (39 %, Sigma) were added. Gels were then poured onto a horizontal gel apparatus (Pharmacia, Milton Keynes, U.K). After removal of the comb, the gel tank was filled

with 1x MOPS buffer until the gel was just covered and the RNA samples were loaded into the wells. 2μl of each of the RNA samples were prepared for electrophoresis by the addition of 5μl/sample of electrophoresis sample buffer [0.75ml deionised formamide, 0.15ml 10x MOPS, 0.24ml of formaldehyde, 0.1ml autoclaved water and 0.1ml of 10% W/V bromophenol blue (Sigma) and 10μl of a 1mg/ml solution of ethidium bromide]. This was then heated at 65°C for 15 min and then cooled on ice prior to loading on the gel. Electrophoretic separation was typically carried out at a voltage of 100V for 40 min and a UV transilluminator used to view the RNA bands.

The intensity of the RNA bands for some of the samples did not appear to correlate with the spectrophotometer readings obtained for them. This can happen as a result of undissolved RNA and so these samples were re-mixed and the process repeated until each sample gave bands of intensity which correlated well with the spectrophotometer readings. Figure 2.1 shows an example of electrophoresed RNA samples from control and orf virus infected keratinocytes, where the intensity of the bands correlate well with the spectrophotometer readings contained within the Table (2.1) below.

**Table 2.1** *RNA spectrophotometer readings*

| Sample 1/20 | abs 260nm | abs 280nm | 260/280nm | 280/260nm | RNA μg/ml |
|---|---|---|---|---|---|
| **blank** | 0.0001 | 0.0002 | 0.836 | 1.1954 | 0.011 |
| **1.** | 0.5552 | 0.2520 | 2.2520 | 0.4535 | 22.190 |
| **2.** | 0.4863 | 0.2189 | 2.2162 | 0.4512 | 19.4897 |
| **3.** | 0.7431 | 0.3375 | 2.1990 | 0.4547 | 29.7569 |
| **4.** | 0.5861 | 0.2648 | 2.2130 | 0.4519 | 23.4437 |
| **5.** | 0.5792 | 0.2616 | 2.2101 | 0.4525 | 23.2065 |
| **6.** | 0.6747 | 0.3101 | 2.1993 | 0.4547 | 26.7414 |
| **7.** | 0.5864 | 0.2668 | 2.2107 | 0.4523 | 23.3381 |
| **8.** | 0.7121 | 0.3265 | 2.1974 | 0.4556 | 28.3369 |
| **9.** | 0.7268 | 0.3366 | 2.1945 | 0.4557 | 28.6704 |
| **10.** | 0.6801 | 0.3166 | 2.1975 | 0.4551 | 26.6841 |
| **11.** | 0.7333 | 0.3486 | 2.1840 | 0.4579 | 28.3882 |
| **12.** | 1.0871 | 0.5201 | 2.1649 | 0.4619 | 43.4878 |

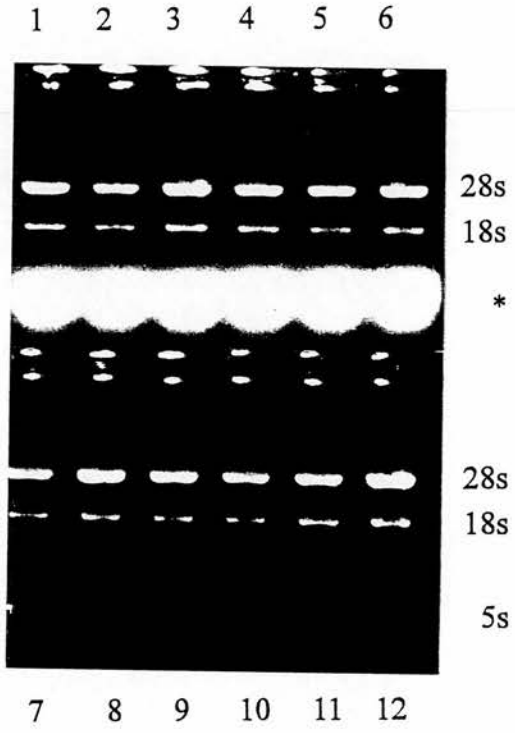



**Figure 2.1** *Whole RNA samples extracted from control and orf virus infected keratinocytes were electrophoresed under denaturing conditions on a 1% formaldehyde/agarose gel. Tracks 1 to 12 contain the RNA samples. *The ethidium bromide has obscured the 5s RNA of the samples in tracks 1 to 7.*

### *iii). cDNA preparation*

cDNA synthesis was primed using a mixture of primers (see Table 2.2) specific for each of the cytokines under study.

The cDNA synthesis reaction was carried out in a total volume of 40μl/sample in individual, sterile eppendorf tubes containing 2μg of each RNA sample in a reaction mixture of reverse transcriptase buffer (50mM TrisHCl, 6mM $MgCl_2$, 10mM dithiothreitol, 100mM NaCl, pH8.2, supplied by Boehringer), 200μM deoxynucleoside triphosphates (dNTPs, Pharmacia), 1μM of each of the 3' primers of ovine specific GM-CSF, TNFα, IL-1β, IL-3 and β actin (Oswel DNA Ltd, Edinburgh, U.K) and 3 units/sample of avian myeloblastosis virus reverse transcriptase enzyme (AMV, Boehringer). The synthesis involved two stages; firstly before the addition of reverse transcriptase, the RNA samples and reaction mixture were incubated at 65 °C for 15 min to denature the RNA. After cooling on ice, 3 units/sample of reverse transcriptase enzyme was added and cDNA synthesis allowed to proceed at 37°C for 90 min. The cDNA was then made up to a total volume of 100μl with sterile distilled water and stored until use at -20°C.

### *iv). Polymerase Chain Reactions (PCR)*

The cDNA samples were all amplified individually for GM-CSF, TNFα, IL-1β, IL-3, and β-actin. PCR reactions were carried out in a total volume of 20μl with 10μl of each cDNA sample in sterile eppendorfs. Individual cytokine-specific reaction mixtures were prepared by the addition of the appropriate primers (3' and 5', see Table 2.2) for the above cytokines to give final concentrations of; 20mmol/l Tris-HCl, 15mmol/l $MgCl_2$, 500mmol/l KCl; pH8.3 (10x PCR buffer Boehringer) and 200μM ultrapure dNTP (Pharmacia) in an appropriate volume of sterile distilled water. Immediately before addition to the samples 1unit/sample of Taq polymerase (Boehringer) was added to the mixture, which was gently mixed and then centrifuged briefly to collect the sample at the bottom of the tube. Approximately 100μl of

Table 2.2 *Cytokine primers and probes*

| Cytokine | Reference | EMBL accession no. | PCR primers | Primer Positions* | Size of fragment (bases) | Position of oligonucleotide probes (40 mers) |
|---|---|---|---|---|---|---|
| IL-1β | 1 | X56972 | 5′ GAACTCATGGCTTACTACAG<br>3′ GTTATATCCTGGCCACCTCT | 28 → 47<br>751 → 770 | 742 | 321- 360<br>583 - 622 |
| IL-3 | 2 | Z18291 | 5′ CGAAGGACCTGGACAAGAACA<br>3′ CCTCAGATCATCAGTGACAAC | - 43 → -23<br>503 → 523 | 551 | 70 -109<br>408 - 447 |
| GM-CSF | 3 | X53561 | 5′ AGTCCTCAAGAGGATGTGGC<br>3′ AAGCTTACCTACCTCACAGA | -13 → 7<br>467→ 486 | 499 | 128 -167<br>409 - 438 |
| TNFα | 4 | X55966 | 5′ TTGCAGGAGCCACCACGCTC<br>3′ TCAGTGCTGAGATCAACCTG | 122 → 141<br>623 → 642 | 520 | 248 - 287<br>471- 511 |
| IFN γ | 5 | X52640 | 5′ CCATAACACAGGACTACCG<br>3′ TCTCAGGGTCCAACTTGGCA | -55 → -36<br>715 → 734 | 789 | 31 - 70<br>301 - 340 |
| β-actin | 6 | # | 5′ TTACAACGAGCTGCGTGTGG<br>3′ AGACTCGTCATACTCCTG | 270→290<br>1077→1094 | 824 | 701-737<br>815-850 |

* primer positions are numbered with respect to the A of the initiator methionine of each sequence.

# approximate positions only

**References:**

1. Andrews et al 1991; Fiskerstrand et al 1990; Seow et al 1990. 2. McInnes et al 1993. 3. McInnes et al 1991; O'Brien et al 1991. 4. Young et al 1990; Green et al 1991; Nash et al 1991. 5. McInnnes et al 1990; Radford et al 1991. 6. Unpublished, personal communication, Colin McInnes.

mineral oil was added to the top of each tube to reduce evaporation.The DNA was denatured at 95°C for 5 min, then amplified through a total of 35 cycles of denaturing, annealing and polymerisation in a Hybaid Omni Gene PCR machine (Hybaid, Teddington, Middlesex, UK) as follows; 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute followed by 1 cycle of 95°C for 1 minute, 50°C for 1 minute and 72°C for 8 min. The samples were then held at room temperature before storing at -20°C.

### *v). Controls*

#### *cDNA reaction*

Approximately 1μg of total RNA prepared from stable Chinese hamster ovary cell (CHO) lines which over-produce the ovine cytokines GM-CSF (McInnes and Haig, 1991) and IL-3 (McInnes and Logan, 1993) were used as positive controls for the reverse transcriptase polymerase chain reactions. Transfectants for the other cytokines were not available.

#### *Polymerase chain reaction*

For each reaction carried out, both negative and positive controls were used. The negative controls consisted of the sterile distilled water used to dilute the samples and reaction mixture. cDNA prepared from the CHO cell lines for GM-CSF and IL-3 was used as the positive controls in the respective reactions. In the absence of a reliable source of ovine IL-1β and TNFα mRNA, positive controls for the PCR stage only were used. These corresponded to 2ng of the cloned ovine cDNA for IL-1β (Seow *et al.*, 1990) and TNFα (Nash *et al.*, 1991).

### *vi). Agarose gel electrophoresis of cDNA Samples*

The PCR cDNA samples were electrophoresed on a 1% agarose gel prepared by dissolving 0.4g agarose in 36mls of 1xTBE (100mM Tris, 100mM boric acid, 1mM EDTA and 0.5 mg/l ethidium bromide) in a microwave oven. Once cool the gel was

poured into a horizontal gel apparatus (Pharmacia) as before. After removal of the comb, the gel tank was filled with 1xTBE until the gel was just covered and the DNA samples loaded into the wells.Four μl of each DNA sample was added to the well with 1.5μl of sample buffer (40% W/V sucrose, 0.25% bromophenol blue in distilled water). On each gel the last well was loaded with 5μl of known molecular weight standards generated from 100μl of lambda phage DNA (500μg/ml, Gibco) digested with 100 units of Hind III. The DNA samples were electrophoresed at a constant voltage of 100V for approximately 40 min. DNA fragments were visualised by a UV light transilluminator and photographed.

The PCR reactions were considered to have worked, when there were no visible bands in the negative controls and the positive controls contained bands of the expected molecular weight for each of the specific cytokines (defined by the primers shown in Table 2.2). Figure 2.2 shows an example of the electrophoresed RT-PCR products for GM-CSF mRNA.

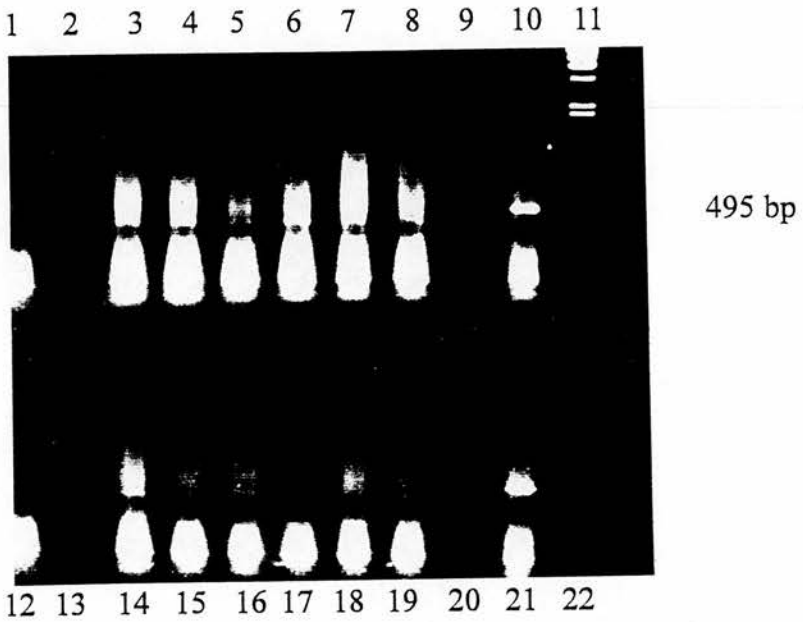



**Figure 2.2** *GM-CSF RT-PCR products from control and orf virus infected keratinocytes electrophoresed on a 1% agarose gel. Tracks 1 and 12 contain the negative control. Tracks 10 and 21 contain the GM-CSF positive control with a single band of 495bp and track 11 contains λ DNA molecular weight markers. The amplified samples are in tracks 3 to 8 and 14 to 19 where some can be seen to contain bands at the expected size for GM-CSF.*

The specificity of detection was increased by blotting the gels onto nylon membranes and probing with cytokine specific labelled probes. The probes hybridise with an internal sequence of DNA contained within the amplified region of each cytokine (see Table 2.2).

### *vii). Immunoblot transfer of cDNA to nylon membranes*

The cDNA was denatured by soaking the gel for 30 min in two changes of a solution of 0.5M sodium hydroxide and 1.5M sodium chloride and neutralised for 1 hour at room temperature in several changes of 1M ammonium acetate and 0.02M sodium hydroxide, both stages with constant agitation. The gel was then placed well-side down on an area of bench covered with saranwrap. A square of nylon membrane (Hybond N, Amersham) cut to the size of the gel was soaked in the ammonium acetate solution and then placed on top of the gel, care being taken at this point to ensure that there were no bubbles breaking the contact of the membrane with the gel. Eight pieces of blotting paper cut to slightly larger than the gel were stacked on top of the membrane, the first four pieces soaked in ammonium acetate, and on top of this a stack of paper towels. The stack was compressed with a heavy book and the transfer allowed to proceed for 16 hours overnight.The stack of towels and filterpapers were then removed and the position of the sample wells on the resulting blot marked in biro and the gel peeled away and discarded. After air drying, the DNA was crosslinked to the nylon membrane in a UV crosslinker (Hybaid) and stored in saranwrap.

### *viii). Digoxygenin Hybridisation*

The membrane bound DNA for each of the PCR reactions was incubated with a specific digoxygenin (Dig) labelled probe. The probes were prepared from oligonucleotides labelled at the 3' end using the enzyme terminal transferase (Boehringer) and the reaction conditions were such that only short tail residues were added to each oligonucleotide. The resulting labelled hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate (antidigoxygenin alkaline phospatase conjugate, <Dig> AP) using the Dig DNA labelling and detection kit

(Boehringer) as follows. The membranes were prehybridised in hybridising solution consisting of; 5x Standard saline citrate (SSC, a 20x stock was prepared by dissolving 175.3g of sodium chloride and 88.2g of sodium citrate in 800mls of distilled water, the pH adjusted to 7.0 with sodium hydroxide and the volume made up to 1 litre), 0.5% W/V Boehringer blocking reagent ; 0.1% W/V N-laurylsarcosine, Na salt and 0.02% W/V sodium dodecyl sulphate (SDS) for 30 min at 55°C in a Hybaid oven. The hybridising solution was then replaced with about 2.5ml per 100cm$^2$ of membrane with hybridisation solution containing approximately 10-50 ng of freshly denatured Dig-labelled DNA probe per ml of hybridisation solution and left to hybridise overnight at 55°C . The probe solutions were then poured off and collected for re-use, any unbound probe was washed off in two washes of 2 x SSC; 0.1% W/V SDS each for 15 min followed by two washes in 0.1x SSC ; 0.1% W/V SDS again for 15 min, all carried out at 55°C. The membranes were transferred into containers on a shaker at room temperature and washed briefly with buffer 1 (100 mM/l Tris-HCl;150mM/l NaCl pH 7.5) and incubated for 30 min in buffer 2 [0.5% W/V blocking reagent (Boehringer) dissolved in buffer 1]. This stage is included to block any non-specific binding of the antibody-conjugate to the membrane. After another 1 minute wash in buffer 1, the membranes were incubated with a 1:5000 dilution (150 mU/ml) of the antibody- conjugate (sheep, polyclonal anti-digoxygenin Fab fragments, conjugated to alkaline phosphatase, Boehringer ) prepared in buffer 1. Any unbound conjugate was subsequently removed by washing the membranes twice in buffer 1 (15 min each). The membranes were equilibrated for 2 min in buffer 3 (100mM/l Tris-HCl; 100mM/l NaCl; 50mM/l $MgCl_2$, pH 9.5) and incubated with 10ml of the colour solution (45µl of 75mg/ml solution of nitroblue tetrazolium salt and 35µl of a 75mg/ml solution of dimethylformamide prepared in buffer 3) in a container kept unshaken in the dark. When the bands appeared (4-6 hours) the reaction was stopped by washing the membrane for 5 min in buffer 4 (10mM/l Tris-HCl; 1mM/l EDTA, pH 8). The membranes were then air-dried and stored wrapped in saranwrap.

## 2.3.3. GM-CSF cytokine protein detection assays

### i). *GM-CSF Antibody capture quantitative ELISA*

A sandwich ELISA developed at the Moredun Research Institute (Entrican *et al.*, 1994) was used for the detection of ovine GM-CSF. The ELISA uses two affinity-purified, neutralising antibodies, 8D8 and 3C2, that recognise mutually exclusive epitopes of GM-CSF. The antibody 8D8 is used to coat the plate and capture any GM-CSF present in the samples and the second antibody 3C2 conjugated to horse radish peroxidase (HRPO) then recognises the second epitope on the captured GM-CSF. The GM-CSF present in the samples is standardised using recombinant ovine GM-CSF (McInnnes and Haig, 1991; McInnes *et al.*, 1993).

Flat-bottomed ELISA plates (Dynatec 129B) were coated with 50µl/well of the anti-GM-CSF affinity purified monoclonal antibody at 1µg/ml in 0.1M carbonate buffer (0.1M sodium carbonate, pH adjusted to 9.6 with 0.1M sodium bicarbonate). The plate was gently tapped to ensure that the whole well was covered and the plates wrapped in clingfilm and left at 4°C overnight. The plates were washed 6 times with wash buffer (PBS/0.05% Tween 20) using a wash bottle and blocked for a minimum of 30 min at room temperature with 50µl/well with 3% bovine serum albumin (BSA, Sigma) in PBS/Tween 20 wash buffer. At this stage the GM-CSF standards were prepared; a stock of recombinant ovine GM-CSF (McInnes and Haig 1991; McInnes *et al.*, 1993) at a concentration of 400µg/ml was diluted to 4000, 2000, 500, 400, 200, 100 and 50pg/ml in 3% BSA/PBS/Tween 20. After the incubation period the plates were washed 6 times with wash buffer and 50µl/well of the standards, samples, or buffer alone were loaded in duplicate to the plates. The plate was left for 90 min at room temperature and then washed 6 times in wash buffer. A 1:800 dilution (of a 0.5mg/ml stock) of the affinity-purified peroxidase-conjugated monoclonal 3C2, was made in wash buffer and 50µl loaded in each well and the plate left at room temperature for 1 hour. After the washing steps the plates were loaded with 50µl/well of O-Phenylenediamine (OPD) substrate (12.85mls 0.2M $Na_2HPO_4$, 12.15mls 0.1M citric acid, 25mls distilled water, 40mg OPD and 40µl hydrogen peroxide) and the

colour reaction allowed to develop. OPD must be stored in the dark and the substrate mixture was prepared immediately before addition to the plate. The reaction was stopped by the addition of 25µl/well of 2.5M $H_2SO_4$ when a gradation of colours could be seen in the standards and the blank wells were still clear (approximately 10 min). The plates were then read at 492nm by a Titretek Multiskan MC.

### *ii). Inhibition of GM-CSF detection by ELISA*

An experiment was designed to determine whether there were any factors released by orf-virus infected keratinocytes that interfered with the detection of GM-CSF in the ELISA. The ELISA was carried out as described previously but before the samples were added to the plate, exogenous GM-CSF at the concentration of 4µg/ml was added to culture supernatants from both uninfected and infected cells. After incubation for 1 hour at 37°C, the samples were diluted serially and added to the plate in duplicate. The ELISA was developed and the adsorption read at 492nm.

Further experiments were carried out to identify the nature of the factor/factors produced by orf virus infected keratinocytes. Before the addition of the exogenous GM-CSF (4µg/ml), the culture supernatants were treated either by heating or by the addition of various protease inhibitors and then the ELISA was carried out as described above. Untreated control and infected samples were included in each ELISA

#### *Heating*

0h, 12h and 24h orf virus infected and control supernatants were heated in a waterbath at 56°C for 1 hour.

#### *Protease inhibitors*

Four types of protease inhibitors; Phenylmethyl sulphonyl fluoride (PMSF), L- trans-epoxysuccinic acid (E64), Pepstatin A and phenanthroline were added individually to both uninfected and orf virus infected 24h supernatants. The targets for the inhibitors and the optimal working concentrations that they were used at, are listed in table 2.3

The supernatants were incubated with the inhibitors for 30 min at 37°C and then the ELISA carried out as described above.

### 2.3.4. Antibody capture quantitative ELISAs for the Interleukins IL-1β and IL-8

Two ELISAs for the detection of IL-1β and IL-8 were used. The method of detection was based on the same principle as the GM-CSF ELISA, i.e. the plate is coated with a monoclonal antibody to the specific cytokine which then captures the cytokine but the second stage antibody is a rabbit polyclonal antibody to either IL-1β or IL-8 which requires a third step, a goat anti-rabbit HRPO labelled antibody to visualise the reaction.

#### *i). Antibodies*

Antibodies for both the IL-1β ELISA and the IL-8 ELISA were gifts from Jim Rothel, CSIRO, Australia.

The antibodies used in the IL-1β ELISA were M IL-1β (monoclonal IgG1 at a 1:200 dilution in 0.05M carbonate buffer) and a second stage polyclonal rabbit anti IL-1β (at a dilution 1:500 in 3% BSA/PBS/Tween 20).

The antibodies used in the IL-8 ELISA were 8M6 (at a 1:200 dilution in 0.05M carbonate buffer) and a second stage polyclonal rabbit anti IL- 8 (at a dilution of 1:2000 in 1.5% BSAPBS/Tween 20).

#### *ii). Procedure*

The ELISA plates were coated overnight at 4°C with the first stage antibody, M IL-1β or 8M6 at the dilutions described above. The coating buffer was then flicked out

Table 2.3 *Protease inhibitors*

| Inhibitor | Protease target | Recommended concentrations µg/ml | Stock solution 1 mg/ml | Working concentration µg/ml | Source |
|---|---|---|---|---|---|
| **PMSF** | Serine proteases | 20-100 | in 1:1/$H_2O$ methanol | 30 | Boehringer |
| **E64** | Cysteine proteases | 0.5-1 | in $H_2O$ | 10 | Sigma |
| **Pepstatin A** | Aspartate proteases | 1 | in methanol | 10 | Sigma |
| **Phenanthroline** | Metalloproteases | 1 | in methanol | 5 | Sigma |

and 100µl of the blocking buffer (3% BSA/PBS/tween 20, described in section 2.3.3 (i)) was added to each well and the plates incubated for 1 hour at room temperature. The plates were then washed four times with PBS/tween 20 (0.05%). 50µl of each supernatant samples and recombinant ovine IL-1β or IL-8 standards were added to the wells in duplicate and incubated at room temperature for 75 min. After washing with PBS/tween 20 (x 4), 100µl of polyclonal rabbit anti- IL-1β or IL-8 sera diluted at the dilutions described above was loaded into each well and the plate incubated at room temperature for 75 min. After a further four washes with PBS/tween 20, 100µl of goat anti-rabbit HRPO diluted 1:1000 in 1% non-specific mouse ascitic fluid (in PBS), to block any non-specific binding, was added to each well and the plate incubated at room temperature for 1 hour. The plates were washed 5 times with PBS/Tween 20 and 50µl/well of the OPD substrate added to the plates and the reaction developed as described for section 2.2.3 (i).

### 2.3.5. Interferon Bioassay

The method used for the detection of interferons (IFNs) was a bioassay developed at the Institute (Entrican, Haig and Norval,1989). The assay measures IFNs by their ability to inhibit virus replication in ovine fibroblasts. The target cells were ovine ST-6 cells, originally cultured from an adenocarcinoma of the small intestine (Norval *et al.*, 1981). The virus used was Semliki Forest virus (SFV) which infects ST-6 cells and induces a CPE after 48h. This procedure was carried out with the assistance of Mary McLean.

***Procedure***

100µl of a suspension of ST6 cells at 5 x $10^3$ cells/ml in growth medium (IMDM supplemented with 5% FBS) was seeded into each well of a flat bottomed, 96 well microtitre plate (Costar, High Wycombe, UK). The plates were incubated for 3 days at 37°C in 5% $CO_2$ humid atmosphere until confluent monolayers had formed. The supernatants to be tested, were filtered through 0.1µm filters (Supor Acrodisc filters, Gelman ) to remove any virus present, as orf virus has been shown to infect ST6 cells

(unpublished data) and would therefore affect the assay. Samples were added to duplicate wells in 100µl volumes and allowed to incubate for 24 hours. After incubation, the medium was discarded from the plates and 200µl of 100 TCID50 of SFV (supplied by Dr. A.G. Morris, University of Warwick) was added to each well and the plates incubated for a further 48 hours. The plate were read visually and the titres expressed as the highest sample dilution which inhibited the cytopathic effect of SFV by greater than 50%.

### 2.3.6. TNFα detection

#### i). *Bioassay for TNFα*

TNFα was monitored using a semi-automated L929 fibroblast cell lytic assay as described by Flick and Gifford (1984). The assay is based on the sensitivity of the transformed murine cell line L929 to TNFα and was carried out with the assistance of Mary McLean.

In brief, L929 TNFα sensitive or TNFα resistant cells were set up in 96 well microtitre plates (Costar) at a density of 1 $x10^5$ cell/ml (100µl/well, in serumless IMDM medium) and incubated overnight at 37°C in 5 % $CO_2$ humid atmosphere until the cells reached sub-confluence. 50µl of each sample was added to duplicate wells, followed by 50µl/well of actinomycin D (in medium, final concentration of 1µg/ml) and the plates incubated overnight. The viability of the cells was checked, the m dium discarded and cell lysis measured by staining the monolayers with crystal violet (0.5%) methanol/water (1:4, V/V). Dye uptake was calculated using an automated micro ELISA autoreader (Titertek, Flow) equipped with a 540nm filter. One unit of TNFα activity was defined as the amount required to lyse 50% of the target cells. An internal standard of positive ovine TNFα from COS-1 transient transfectant supernatant (ovine TNFα cDNA, Egan *et al.,* 1994) was included in each assay.

### ii). *Radioimmunoassay for TNFα*

A radioimmunoassay for the detection of bovine TNFα that has also been shown to detect ovine TNFα was developed at the Moredun Research Institute, following a method based on that reported by Kenison *et al.,* (1990). The method used recombinant bovine TNFα (rbo TNFα) cloned and expressed in Escherichia coli (provided by Gary Entrican, MRI, obtained from CIBA-GEIGY Ltd, Switzerland), and anti-bovine polyclonal antiserum also produced at MRI. The procedure was carried out on my behalf by Gordon Moon. In brief, the assay was performed in 75 x 11mm polystyrene tubes (Sarstedt). 50µl of standards (rbo TNFα), controls or test samples were mixed with 50µl polyclonal antiserum (1:4000) and 50µl of assay buffer (0.15M PBS pH 7.4, containing 1%BSA) and incubated for 24 hour at 4°C. At the end of this period 50µl of radiolabelled TNFα (prepared by the iodogen method, Peel *et al.,* 1990) was added to all the tubes which were vortexed and incubated for a further 18h at 4°C. 200µl of the second antibody; donkey anti-rabbit IgG (SAPU), was then added to all the tubes except total count (TC) tubes (contain radiolabelled TNFα only) and incubated for 2h at 4°C. To enhance pellet formation, 50µl of 1% rabbit serum (SAPU) was added to the tubes and the incubation continued for a further 30 min. All tubes, except Tc, were then centrifuged (383g) for 15 min and the supernatant decanted. The radioactivity in the pellet was measuerd in a gamma counter (Packard Auto-Gamma 5650). Results were analysed using an in-house RIA programme.

## 2.3.7. Analysis of the proteolytic activity of keratinocytes

The supernatants and cell lysates collected for the analysis of $^{35}S$ methionine incorporation (previously described) were also analysed for proteolytic ativity. The cell-free supernatants were concentrated 10-fold in a Centricon 10 micro-concentrator cell (Amicon, Stonehouse, U.K) at 6000 x g for 3 hours. Supernatant and cell lysate samples were loaded onto 5 %, 12 % discontinuous SDS page gel containing 0.2 % (W/V) azocasein (Sigma) in the separating gel component and the gels run as above but under non-reducing conditions (in sample buffer without 2-mercaptoethanol and

without boiling). Once run, the substrate gel was washed in 2 volumes of 2.5% Triton X100 in water for 2 hours, then briefly in water and finally in PBS before an overnight incubation at 37°C. Digestion of the substrate was detected by staining the gel with Coomassie brilliant blue and identifying clear areas.

# CHAPTER 3

## PHENOTYPIC CHARACTERISATION OF THE DENDRITIC CELLS ACCUMULATING IN THE OVINE DERMIS FOLLOWING PRIMARY AND SECONDARY ORF VIRUS INFECTIONS

## 3.1. INTRODUCTION

Dendritic cells form a system of highly specialised antigen presenting cells (APC) that are widely distributed in the body (Steinman, 1991). In the skin, two populations of dendritic cells exist; the epidermal Langerhans cell (LC) and the dermal dendritic cell. Epidermal LC represent the best characterised APC. They function as sentinels in the skin and are important in inducing immunity to the foreign antigens encountered there (Romani and Schuler, 1992). LC have been shown to be involved in the pathogenesis of a number of infections of viral origin (Sprecher and Becker, 1988). Dermal dendritic cells, recently identified by Headington (1986), are a poorly defined population of cells of bone marrow origin. They lack the LC associated antigens such as CD1 and the distinctive organelle, the Birbeck granule and thus represent a population of cells distinct from LC. Dermal dendritic cells have recently been characterised as being positive for the antigen factor XIIIa and are increasingly being recognised as having a role in the induction of cutaneous immune responses (Cerio *et al.*, 1989).

Orf virus enters through abrasions in the skin where it replicates in the regenerating epidermal keratinocytes (Jenkinson *et al.*, 1990a). Orf is an acute, localised infection that has no systemic phase. The localised nature of the infection and the ability of the virus to reinfect despite the presence of specific anti-viral antibodies, highlights the importance of the local, cellullar immune response in controlling the infection. The host cutaneous response to orf virus infection involves the accumulation of a large number of neutrophils and lymphocytes adjacent to and underlying the infected epidermis (Jenkinson *et al.*, 1990a). A secondary infection is characterised by an accelerated response resulting in a much milder infection which resolves faster (Jenkinson *et al.*, 1990b). As LCs play a role in the control of infections of the skin with viruses such as herpes simplex virus-type 1 and vaccinia virus (Sprecher and Becker 1986, 1988 and 1991), they were postulated to have an important role in the immune response to orf virus infection. Jenkinson *et al.*, (1991) showed that a dense accumulation of MHC class $II^+$ dendritic cells formed at the site of the orf lesion.

These dendritic cells were hypothesised to form a barrier to viral invasion and a template for subsequent epidermal repair. However they lacked the ovine LC marker acetylcholinesterase (AchE), thereby sharing the phenotype of the $AchE^-$ dendritic cells found in the dermis of normal ovine skin. It was proposed that these $AchE^-$ dermal dendritic cells were likely to represent a heterogeneous population of cells that may be differentially involved in the MHC class $II^+$ dendritic cell response to orf virus infection (Jenkinson *et al.*, 1991).

In this chapter, sheep were infected with orf virus following scarification and skin biopsies were collected at various times thereafter. These were examined by immunocytochemistry using a range of specific antibodies. Comparisons were made between the dendritic cell populations in primary and secondary orf virus infection, control scarification and normal ovine skin.

The aims of the study were three-fold;

1. Using the dendritic cell markers CD1 and factor XIIIa, we wished to further characterise more fully the $AchE^-$ dendritic cells and in particular, to determine whether the dendritic cells, accumulating adjacent to or below the orf lesion, differ phenotypically from those normally present in uninfected ovine skin.

2. To establish whether there is any relationship between the accumulating dendritic cells and cells of the monocyte-macrophage lineage.

3. To determine whether the dendritic cells proliferate locally.

## 3.2. RESULTS

### 3.2.1. Clinical observations and serology

*i). Control scarification*

The lines of scarification in the control, uninfected group developed scabs by 24h, with a mild associated erythema. By 48h the scabs were beginning to detatch, and at 72h only a fine, non-inflamed scar was visible. No swelling or vesicles, indicating any secondary infection of the animals, was evident.

*ii). Primary orf virus infection*

Each of the four SPF lambs developed all the typical phases of an experimental orf virus infection; macule, papule, vesicle, pustule and the formation of a scab which was shed on resolution of infection. The initial erythematous response observed in the first 24 to 48 hours could not be distinguished from the scarification controls and was due to the trauma of scarifiying the skin. Vesicles and pustules, approximately 2mm in diameter, developed along the scarification lines at day 3 (72h) and day 6 (148h) respectively and by day 9 scab formation was observed on all four sheep. In 3 of the 4 sheep detachment of the scab was observed after 22 days, however the fourth animal still had profound lesions after 30 days.

*iii). Secondary orf virus infection*

The stages of papule, pustule, scab formation and resolution of infection were observed in 3 of the 4 sheep. The lesions developed earlier (pustules appeared at 48-72h) and resolved faster than the primary challenge; scabs forming by 96h and beginning to be shed by day 7 (170h). The fourth animal did not develop any visible evidence of an orf infection, but subsequent studies, described below, showed that the animal was successfully infected with orf virus, although it was a milder infection.

*iv). Serology*

The four animals used in the secondary challenge had been conventionally raised and as they were over the age of 1 year were expected to have been exposed to natural orf infection in the field. Previous exposure to orf virus was confirmed by screening serum taken from the animals prior to the start of the experiment using an ELISA specific for orf virus. The sera of all four animals showed high titres of antibodies to orf virus indicating recent exposure. The fourth animal that did not develop an obvious lesion (mentioned above), did not have an antibody titre that differed significantly from the other three animals.

## 3.2.2. Immunohistochemistry

The results for each of the markers are described individually and subdivided into four groups; normal skin, scarified skin, primary orf virus infection and secondary orf virus infection.

*i). Dendritic cell phenotype.*

*Normal skin*

Four populations of MHC class $II^+$ dendritic cells were observed in normal skin. Their phenotypes are summarised in Table 3.1.

**Table 3.1** *The dendritic cell populations of normal skin*

| markers/cells | MHC class II | AchE | CD1 | factor XIIIa | %* |
|---|---|---|---|---|---|
| Epidermal LC | + | + | + | - | |
| Dermal DC (1) | + | - | + | - | 45 |
| Dermal DC (2) | + | - | - | + | 15 |
| Dermal DC (3) | + | - | - | - | 40 |

* Each population as a percentage of the total MHC class $II^+$ dendritic cells present within normal skin.

$CD1^+$ dendritic cells were seen within the epidermis generally at, or immediately above, the *stratum basale* and within the dermis clustered at the dermal/epidermal junction or closely associated with dermal structures such as sweat glands, hair follicles and blood vessels. A comparison between dendritic cells expressing MHC class II and CD1 antigens in serial sections (Figure 3.1A and B) showed that all $CD1^+$ dendritic cells coexpressed class II antigens. Within the epidermis all dendritic cells were $CD1^+$ and MHC class $II^+$, but only 50% of the MHC class $II^+$ dendritic cells in the dermis were positive for CD1. A small population of factor $XIIIa^+$ dendritic cells were observed within the dermis, typically at the dermal/epidermal junction (Figure 3.1C) and surrounding blood vessels, particularly in the *papillary dermis*. Analysis of serial sections showed that factor $XIIIa^+$ dendritic cells were $CD1^-$ and coexpressed MHC class II. However, they represented only a small proportion (approximately 15%, Table 3.1) of the MHC class $II^+$ dendritic cells present within normal ovine skin.

*Scarified skin*

A significant increase of MHC class $II^+$ dendritic cells and factor $XIIIa^+$ dendritic cells from the number found in normal skin was observed at 48h, following scarification ($p < 0.05$ and $p < 0.01$ respectively, Figure 3.2). The populations accumulated transiently at the dermal/epidermal junction on either side of the abrasion at 48h (Figure 3.3A and B). Some diffuse staining, that did not appear to be cell-associated, was observed with factor XIIIa at the very edges of the lesion and also within the scab. $CD1^+$ dendritic cells were absent from the dendritic cell accumulation and were found only in the undisturbed dermis/epidermis adjacent to the abrasion. At 96h $CD1^+$ dendritic cells had appeared along the boundary of the new epidermis and by 170h their distribution in the epidermis and dermis had returned to that seen in normal, uninfected skin.

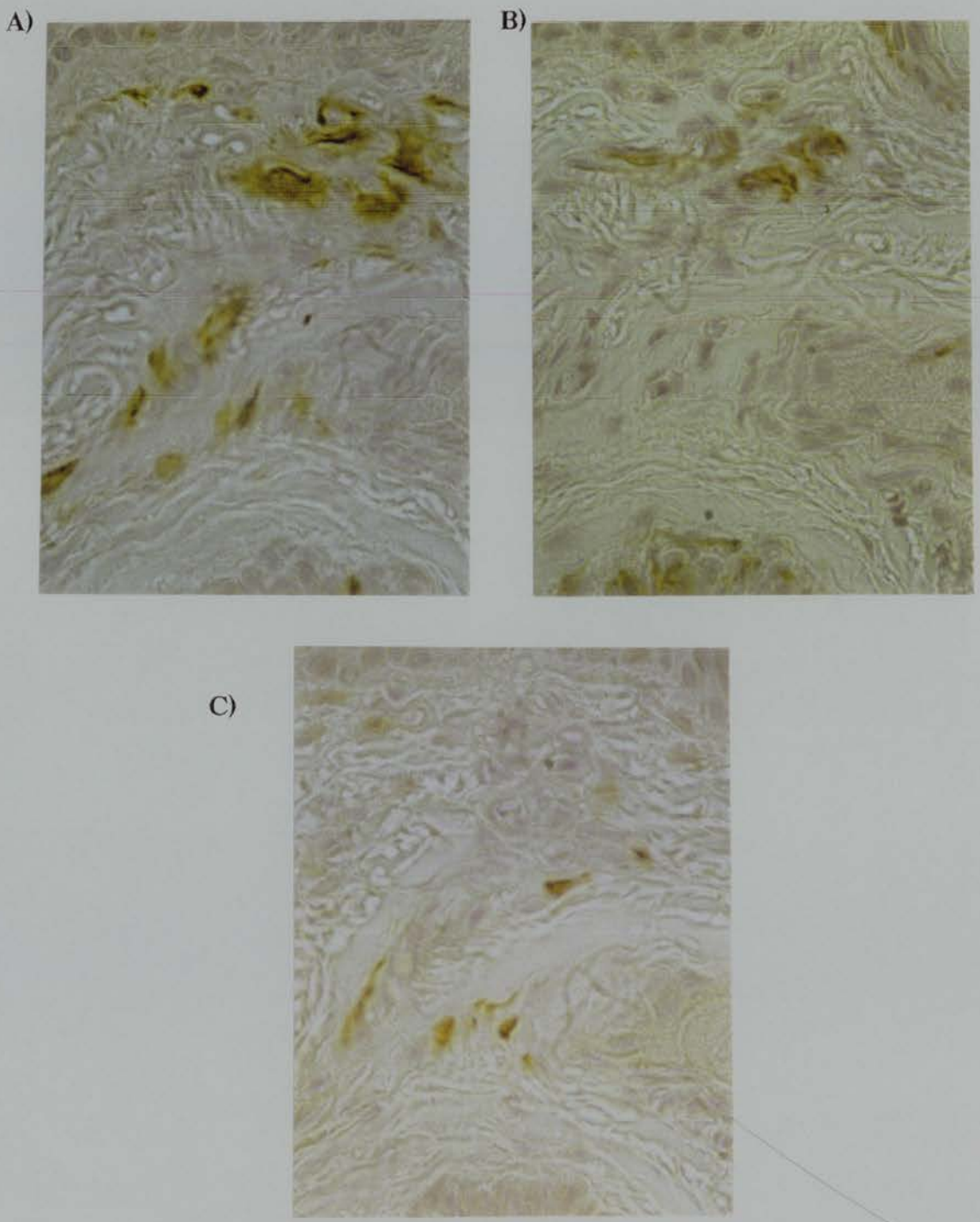



**Figure 3.1** *Serial sections of normal ovine skin showing the distribution of* **A)** *MHC class II$^+$ dendritic cells* **B)** *CD1$^+$ dendritic cells and* **C)** *Factor XIIIa$^+$ dendritic cells. Immunoperoxidase* **X 560**

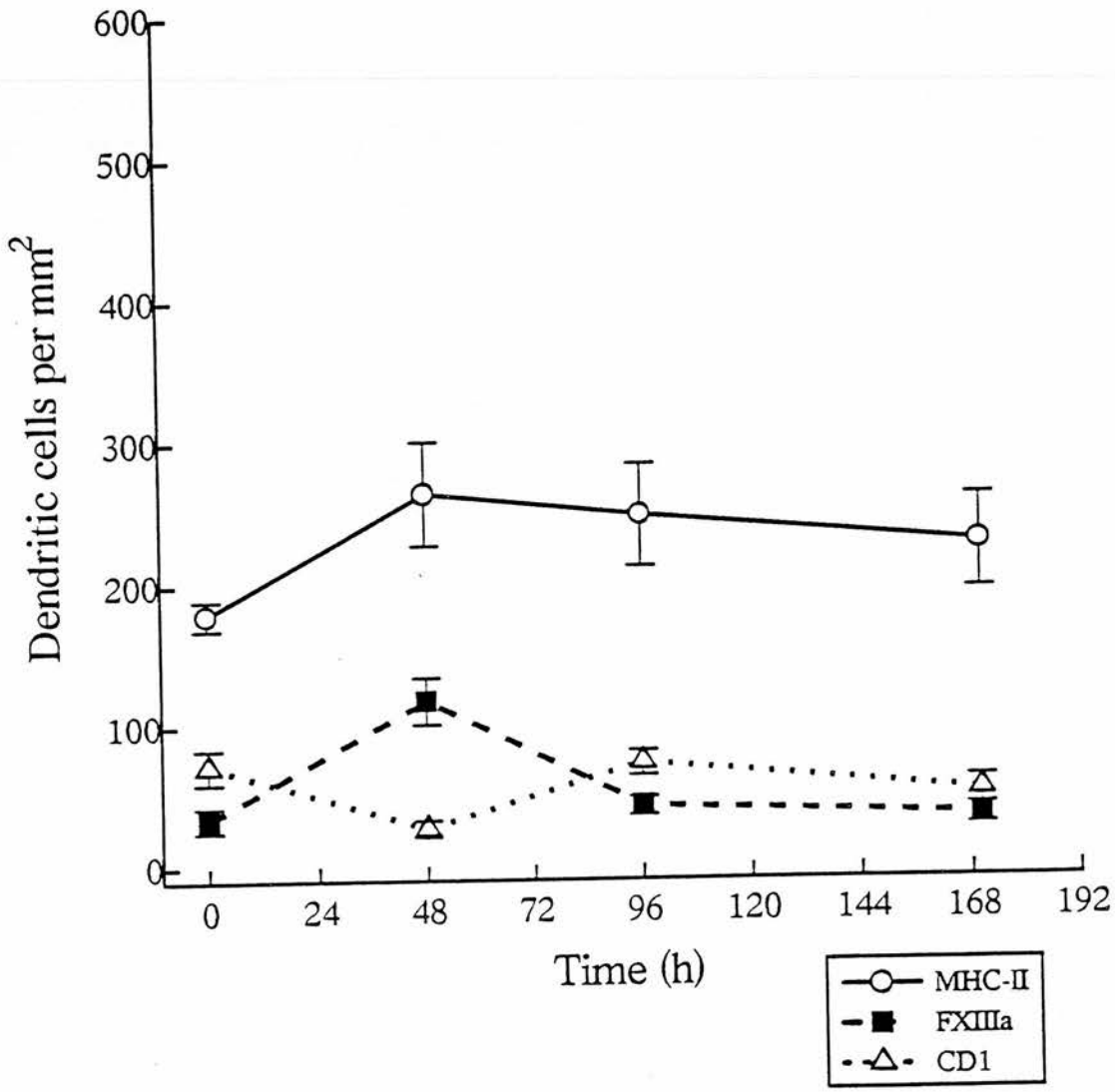
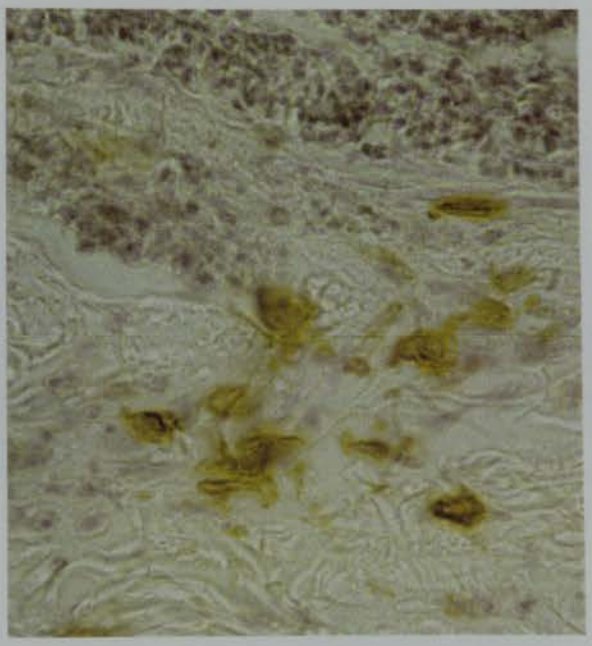



**Figure 3.2** *Changes in the MHC class II$^+$, CD1$^+$ and factor XIIIa$^+$ dendritic cell populations of the skin in response to scarification.*

A)



B)

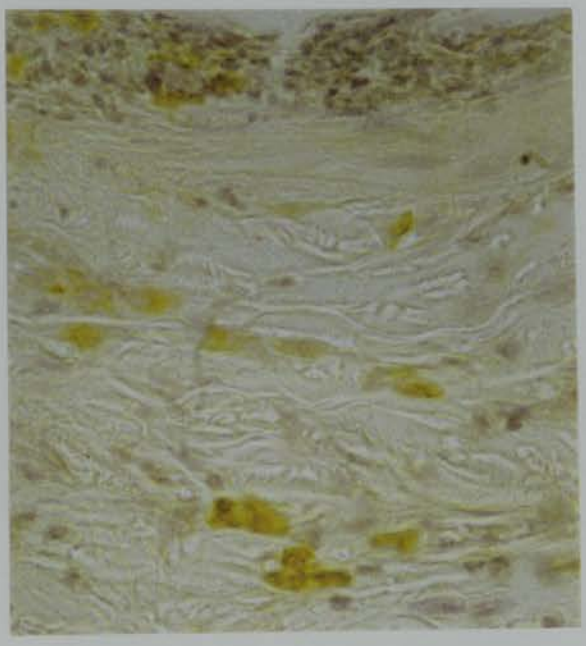


**Figure 3.3** *Serial sections of ovine skin, 48h post-scarification, showing the accumulation of* **A)** *MHC class II$^+$ dendritic cells and* **B)** *factor XIIIa$^+$ dendritic cells beneath the damaged epidermis. Note the diffuse staining of factor XIIIa at the edges of the abrasion. Immunoperoxidase* **X 560**

*Primary Orf virus infection*

The dendritic cell response at 48h post-infection was similar to the response observed for scarification, with an increase in MHC class II$^+$ and factor XIIIa$^+$ dendritic cells. From 48h onwards the MHC class II$^+$ dendritic cells continued to accumulate forming a dense network beneath the infected, degenerating epidermis (Figure 3.4 and Figure 3.5A) and clustered between epidermal down growths. The dendritic cell accumulation reached a maximum between days 9 to 12 and then decreased returning to a pre-infection distribution by day 30 (not shown). Factor XIIIa$^+$ dendritic cells accumulated in high numbers at the lesion (Figure 3.4 and Figure 3.5B), forming a significantly high proportion of the dendritic cell network at 170h when compared with the equivalent time point in the scarified only skin (53%, $p < 0.0001$). The accumulation of factor XIIIa$^+$ dendritic cells was restricted to the dermis (Figure 3.5C), unlike the MHC class II$^+$ dendritic cells which could be seen infiltrating the infected epidermis (Figure 3.5D). Although 170h post-infection was the last timepoint for cell enumeration, qualitative analysis thereafter showed that factor XIIIa$^+$ dendritic cells persisted until day 12, clustered between the epidermal downgrowths, and then declined in parallel with the MHC class II$^+$ dendritic cells. The dense accumulation of dendritic cells was negative for CD1 at all the timepoints studied. With the onset and spread of infection (from 48h post-infection), CD1$^+$ dendritic cell numbers decreased (Figure 3.4), and by 170h these cells were virtually absent from the skin when compared with the same timepoint of the control scarified skin ($p < 0.015$). On resolution of infection (day 16, as shown by disappearance of viral antigen) CD1$^+$ dendritic cells reappeared at the epidermal/dermal junction and subsequently within the thickened epidermis on day 22.

*Secondary orf virus infection*

The MHC class II$^+$ dendritic cell response that occurred within the first 96h after a secondary challenge with orf virus was similar to the response observed during this time following primary infection (Figure 3.6). The dendritic cell network was densest at 96h after which cell numbers declined as the infection resolved (170h, not shown). Which is consistent with previous findings (Jenkinson *et al.*, 1991). Factor XIIIa$^+$

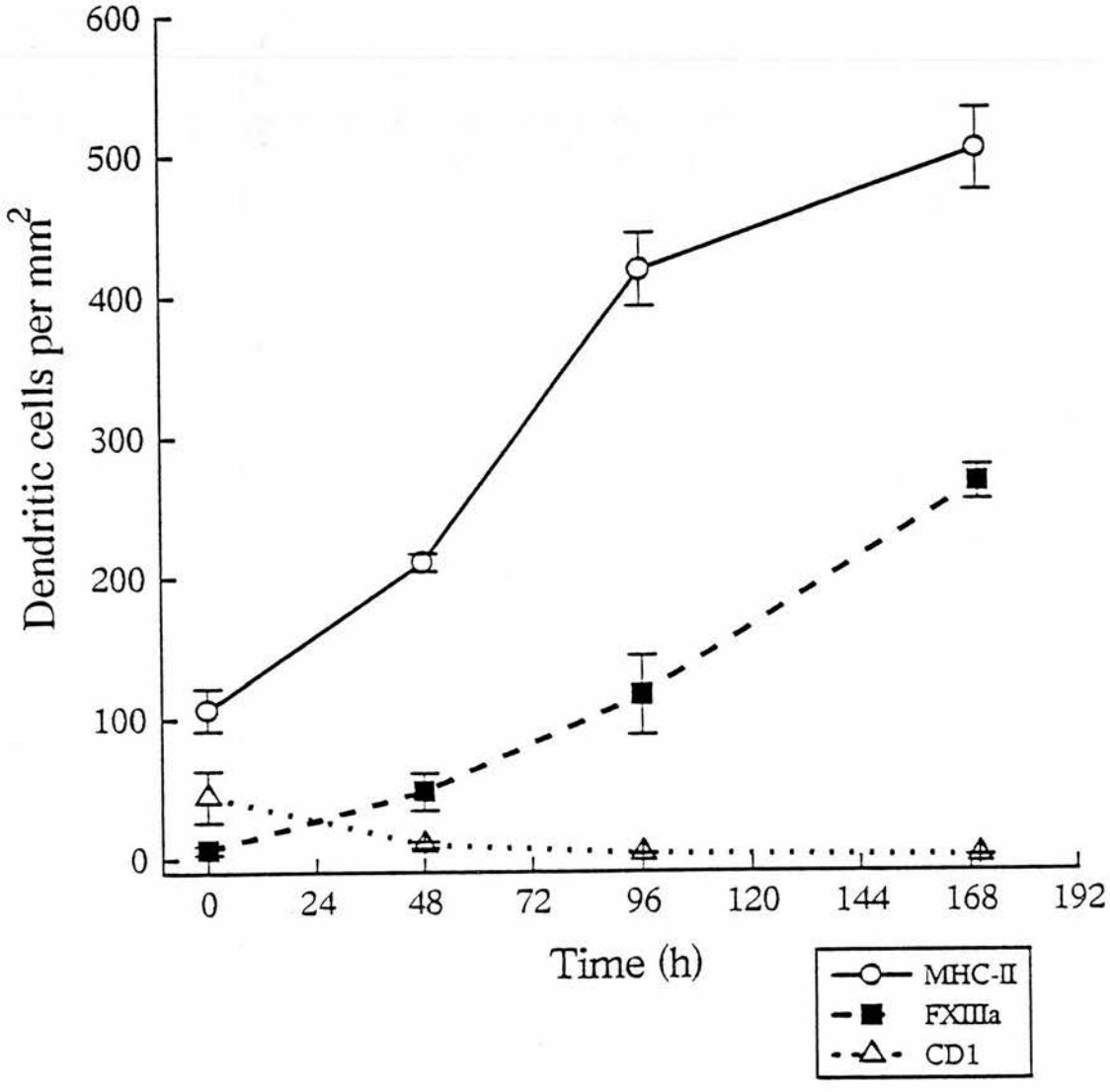
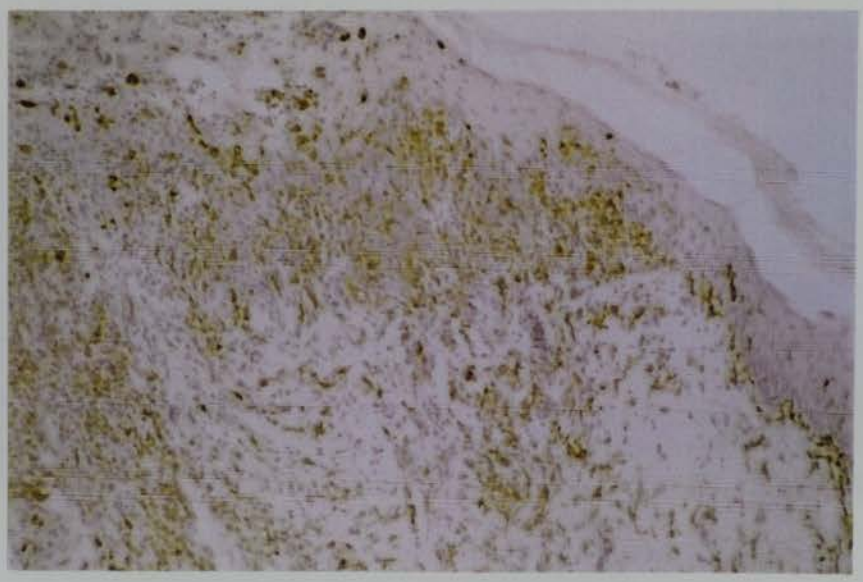



**Figure 3.4** *Changes in the MHC class II$^+$, CD1$^+$ and factor XIIIa$^+$ dendritic cell populations of the skin in response to a primary infection with orf virus.*

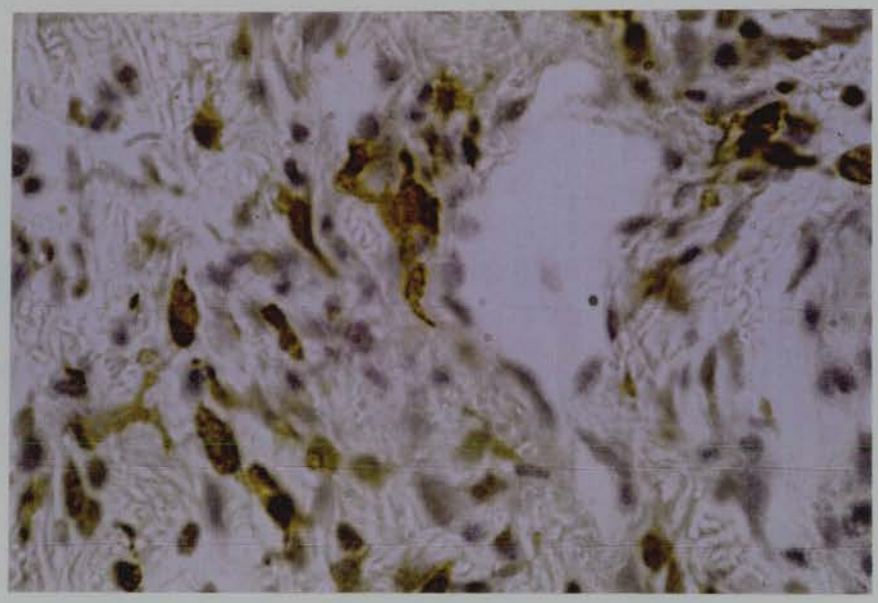
A)



B)

**Figure 3.5** *Serial sections of ovine skin 170h post-primary infection with orf virus, showing the extensive nature of the lesion, with the ballooning degeneration of the epidermis and the accumulation of* **A)** *MHC class II*$^{+}$ *dendritic cells and* **B)** *factor XIIIa*$^{+}$ *dendritic cells. Immunoperoxidase* **X 112**.

C)



D)

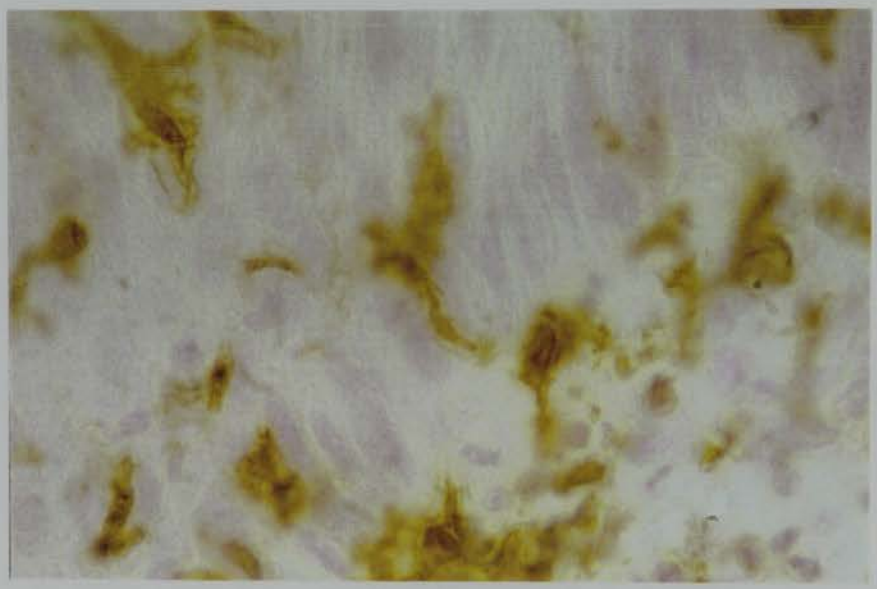


**Figure 3.5** *High power view of the dendritic cell nature of* **C)** *the factor XIIIa*$^{+}$ *dendritic cells gathered at the site of a primary orf lesion, where they are restricted to the dermis and* **D)** *the class II*$^{+}$ *dendritic cells which infiltrate into the infected epidermis. Immunoperoxidase* **X 560**

dendritic cells peaked transiently at 48h, as observed for control scarification group, and were not present in the dense network of MHC class II$^+$ dendritic cells at 96h, representing only 1% of the total MHC class II$^+$ dendritic cells present in the section. However their numbers had increased by 170h (reaching 10% of the total MHC class II$^+$ dendritic cells present) as the infection started to regress. CD1$^+$ dendritic cells were absent from the lesion and their numbers decreased as the lesion extended into previously unaffected dermis/epidermis. At 170h the occasional CD1$^+$ dendritic cell was present at the dermal/epidermal junction of the healing lesion.

### *ii). Anti-proliferating cell nuclear antigen (PCNA)*

#### *Normal skin*

PCNA$^+$ cells were present in low numbers in the normal skin of all the animals studied. Typically the positive cells were observed in the basal layer of the epidermis and surrounding sweat glands and at the base of hair follicles (Figure 3.7A). These cells did not have a dendritic cell morphology.

#### *Scarified skin*

Intense PCNA staining was observed at 48h post scarification in the epidermal cells flanking the edges of the newly regenerating epidermis (Figure 3.7B). The cells infiltrating the dermis beneath the lesion were negative for PCNA. The newly formed epidermis also contained some positive cells at 96h and by 170h the skin resembled the uninfected state, with only faintly staining PCNA$^+$ cells detectable.

#### *Primary orf virus infection*

At 48h post infection, PCNA staining was similar to that seen at the same time following scarification only, being focused at the edges of the lesion and in the newly formed epidermis. The proliferative activity within the epidermis decreased after 96h when the epidermal cells at the edges of the expanding lesion became vacuolated due to viral replication and only faint staining with PCNA was detectable. By 170h PCNA

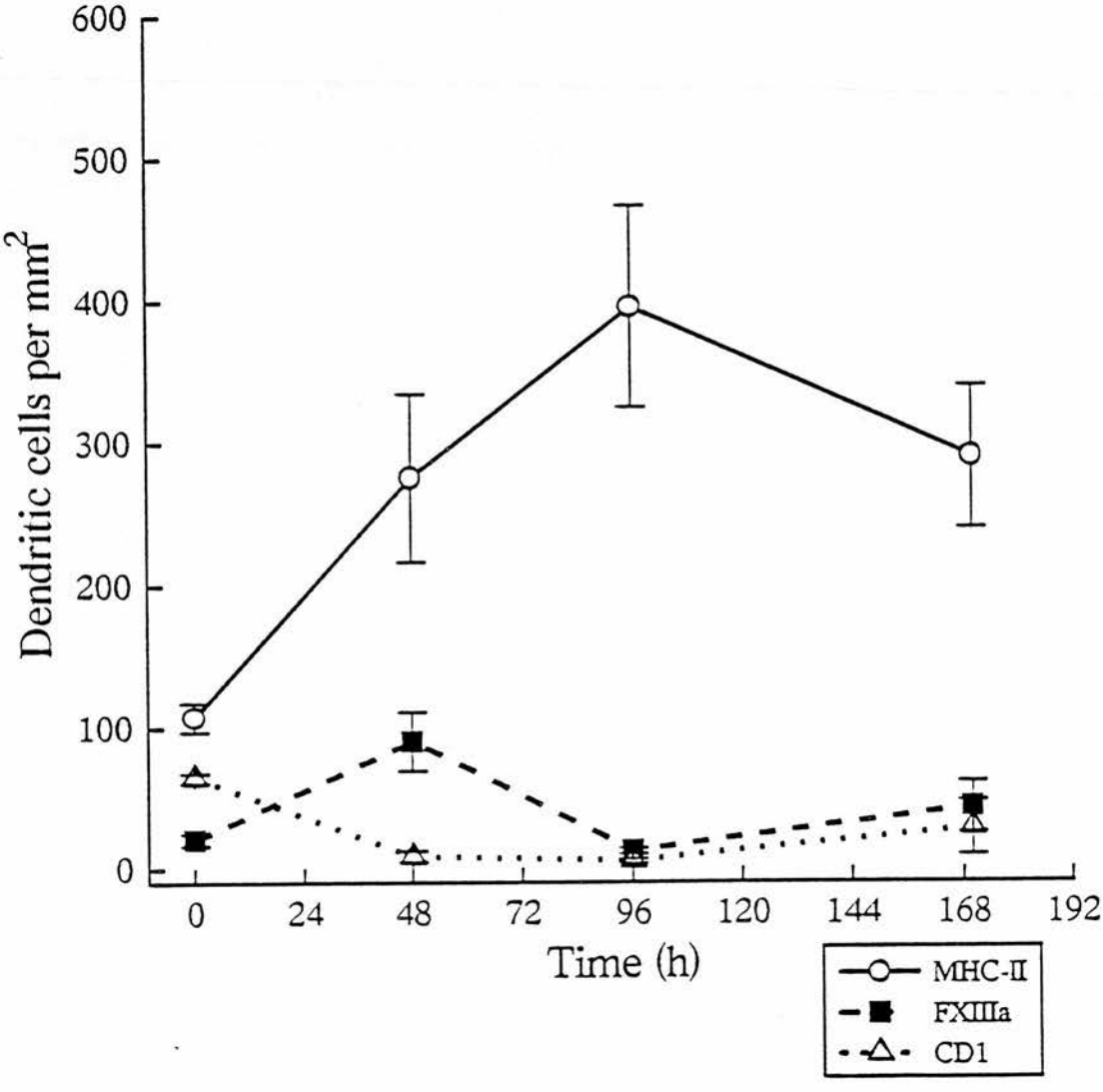



**Figure 3.6** *Changes in the MHC class II$^+$, CD1$^+$ and factor XIIIa$^+$ dendritic cell populations of the skin in response to a secondary infection with orf virus.*

staining began to increase and intense nuclear staining was observed within the developing epidermal downgrowths (Figure 3.8A), which increased in size over the next few days (days 9 to 12, not shown). Positive cells were also present within the dermal cellular influx (48h onwards). Dual staining of these cells with MHC class II revealed that a few of the proliferating cells were class $II^+$ dendritic cells (Figure 3.8B). By days 22 to 30 $PCNA^+$ cell numbers decreased and the skin returned to a normal level of pre-infection PCNA staining.

*Secondary orf virus infection*

The pattern of cellular proliferation was similar to the scarification already described, with positive cells flanking the edges of the lesion at 48h. Proliferation was not observed at any time within the dermal influx of dendritic cells; and the dense accumulation of cells at 96h was completely negative for PCNA staining. Epidermal downgrowths were not observed and the only proliferating cells that were present were within the healing epidermis (at 170h).

*iii). Monocyte-macrophage markers*

As the monoclonals which recognise monocytes/macrophages had not been fully characterised on ovine cells, they were first used on alveolar macrophages and bone marrow cells from sheep. The monoclonals used were OM1, OM2, OM3 and IL-A15 and IL-A24.

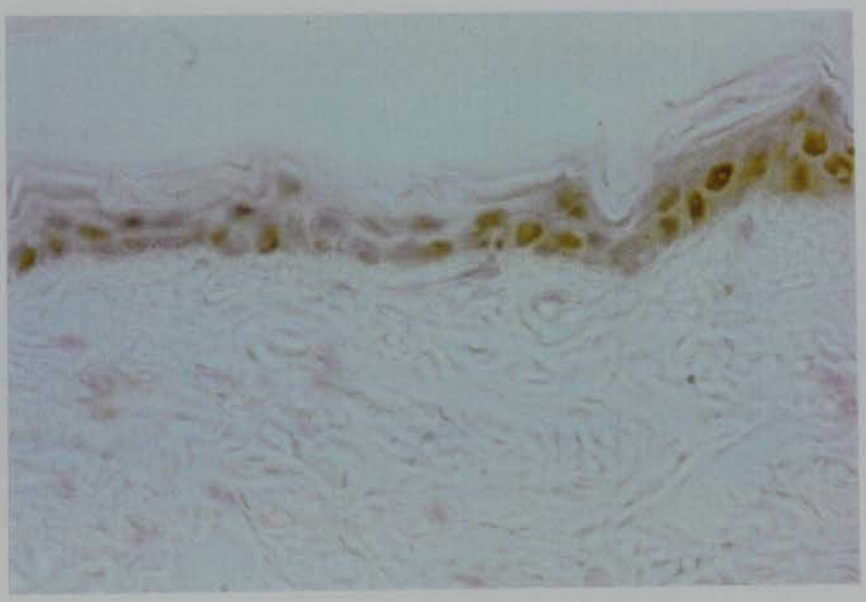
*Alveolar cytospins*

The three OM monoclonals gave strong staining of the alveolar macrophages with the most intense staining observed for OM3 (Figure 3.9A). The IL-A antibodies, giving fainter and more variable staining, did not recognise all of the macrophages.

*Bone Marrow cytospins*

The three OM monoclonals reacted with only a few of the cells in the bone marrow cytospins. These cells had macrophage-like morphology. The IL-A antibodies

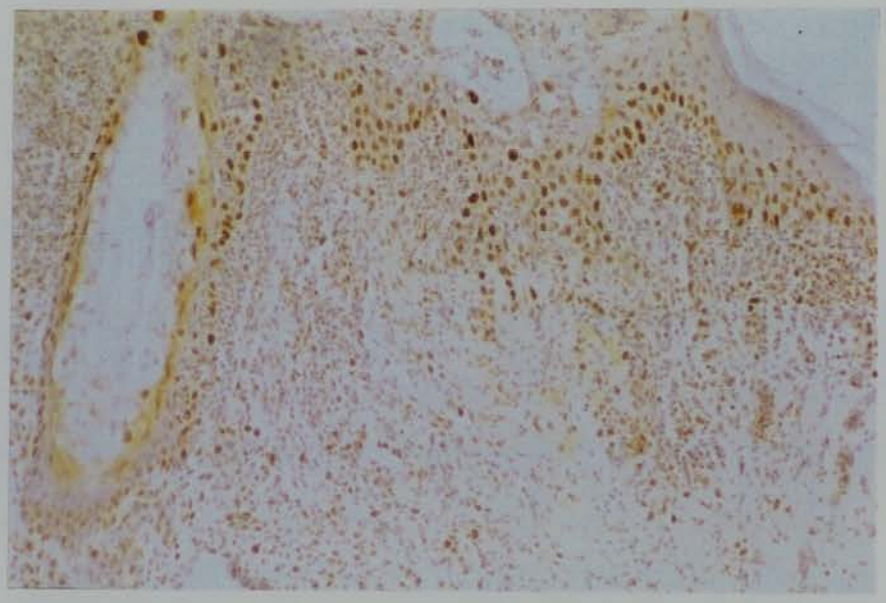
A)



B)

**Figure 3.7** *The proliferative response of ovine skin* **A)** *under normal 'resting' conditions, where some epidermal keratinocytes are positive for PCNA, Immunoperoxidase* **X 560** *and* **B)** *at 48h post-scarification, where PCNA positive cells can be seen to line the edges of abraded skin and represent the epidermal keratinocytes involved in the healing process. Immunoperoxidase* **X 112**

A)



B)

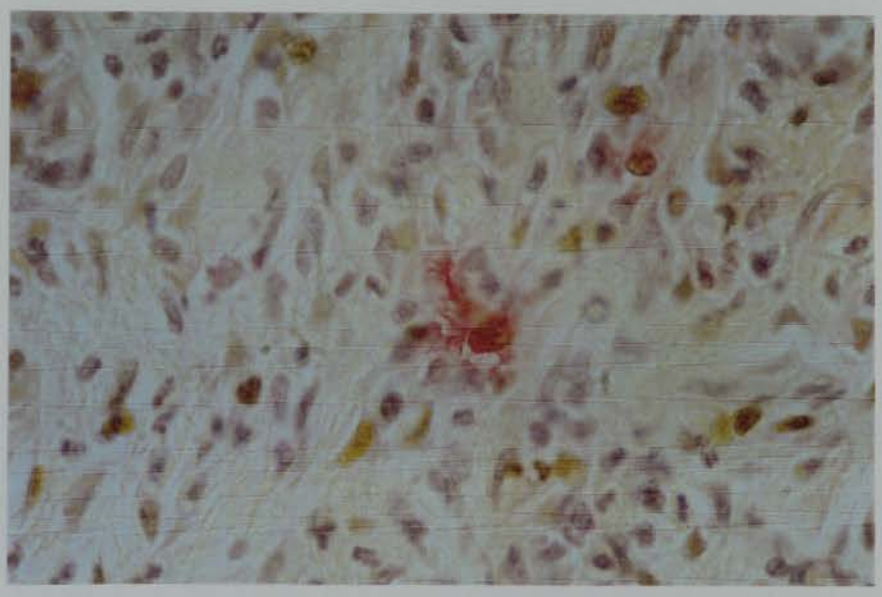


**Figure 3.8** *The proliferative reponse of ovine skin 170h post-primary infection with orf virus .* **A)** *A large number of intensely PCNA positive cells are seen in the developing epidermal downgrowths and surrounding infected hair follicles and within the dermal influx. Immunoperoxidase* **X112**

**B)** *Double staining with MHC class II (pink) and PCNA (brown) shows a proliferating MHC class* $II^+$ *dendritic cell within the inflammatory influx of cells. Immunoperoxidase and alkaline phosphatase* **X 560**

stained a larger proportion of the cells. IL-A15 stained neutrophils strongly and a population of pro-monocytes. IL-A24 stained monocytes and the more mature neutrophils and reacted slightly with a population of macrophages (Figure 3.9B).

*Normal skin*

Few cells within normal skin stained with the OM monoclonals. The occasional positive cell that had macrophage-like morphology could be detected within the dermis, typically associated with dermal structures such as sweat glands. The OM2 monoclonal also recognised endothelial cells lining blood vessels in the skin. IL-A15 gave very similar results, with only a few positive macrophage-like cells detectable within the dermis. IL-A24 stained cells with dendritic processes in the epidermis and surrounding the tops of hair follicles.

*Scarified skin*

Scarification of the skin generated a small influx of neutrophils along with the MHC class $II^+$ cells. The OM monoclonals did not recognise either the neutrophils or the MHC-class $II^+$ dendritic cells. Both IL-A15 and IL-A24 monoclonals did show some staining but this was of neutrophils and not class $II^+$ cells. No macrophage-like cells were observed to react with any of the markers used.

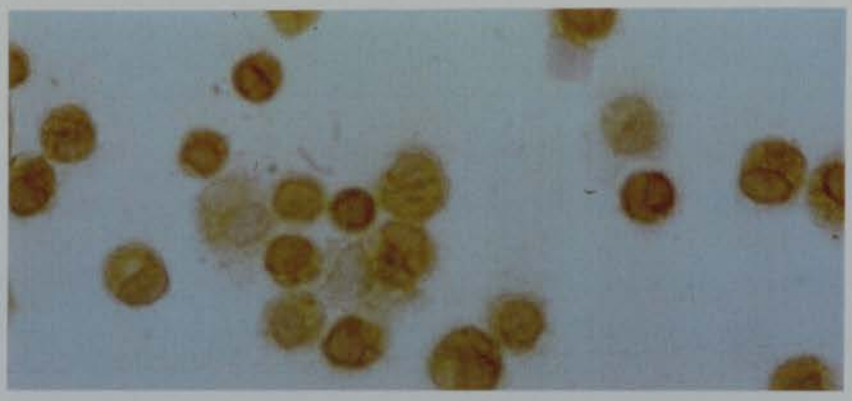
*Primary Orf virus infection*

The primary lesions formed quite extensive pustules beneath the scab and the high neutrophil content within them stained intensely with IL-A15 and IL-A24 (Figure 3.9C). The OM monoclonals gave no staining with any of the cell types at any of the timepoints observed. No macrophages or dendritic cells were observed staining with any of the markers.

*Secondary orf virus infection*

The IL-A antibodies did not stain any dendritic cells present at any of the timepoints following infection and the positive cells that were present in high numbers were the

A)



B)

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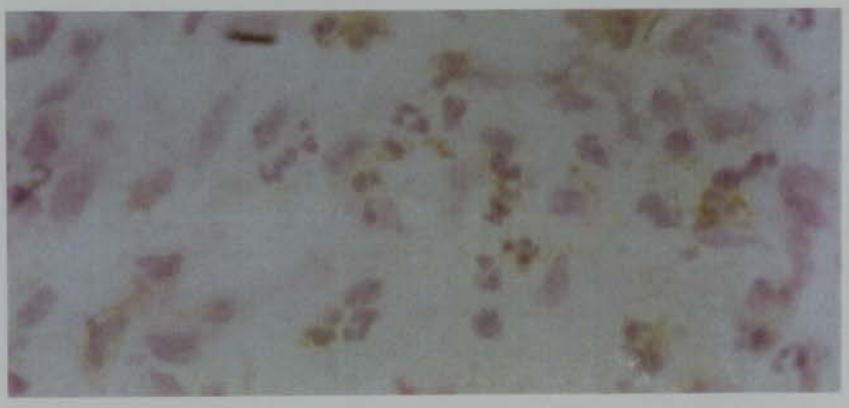


**Figure 3.9** *Antibodies specific for monocyte-macrophages.* **A)** *showing the intense staining of ovine alveolar macrophages with the antibody OM3 and* **B)** *IL-A24 positive cells within a preparation of ovine bone marrow cells.* **C)** *shows IL-A24 positive polymorphonuclear neutrophils within the inflammatory influx at 96h post-primary infection with orf virus. No positive dendritic cells are visible. Immunoperoxidase* **X 560**

neutrophils. The OM monoclonals did not stain any of the cells in the dermis at any time post-infection.

### *iv). Orf virus antigen*

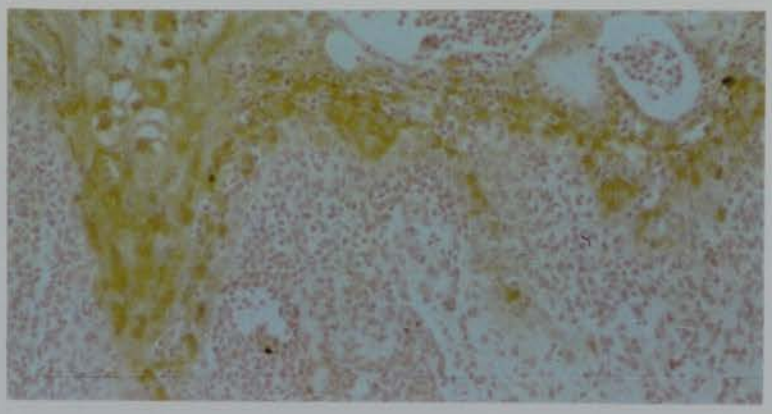
#### *Normal skin/ Scarified skin*

Viral antigen was not detected in the sections of normal, uninfected skin or in the control scarified group.

#### *Primary orf virus infection*

The regenerating epidermal keratinocytes, positive for PCNA at 48h post-scarification and infection were negative for orf viral antigen. From 72h onwards these epidermal cells became vacuolated and positive for orf viral antigen. As the infection progressed (170h onwards), viral antigen spread laterally and intense staining of the damaged, vacuolated cells of the epidermis was seen (Figure 3.10A). The cells containing viral antigen were negative for PCNA, but the epidermal keratinocytes directly beneath them were intensely $PCNA^+$ (Figure 3.10B). It was directly below these two layers of viral antigen and $PCNA^+$ cells that the factor $XIIIa^+$ dendritic cells were gathered (Figure 3.10C). By day 12, orf viral antigen was no longer restricted to the outer edges of the lesion and had spread to encompass hair follicles and was observed in areas of degenerating epidermis in between $PCNA^+$ epidermal downgrowths. On day 16 viral antigen was detectable within the epidermal downgrowths, which had lost their intense PCNA staining, but were surrounded by high proliferative activity. By day 22 only one sheep still had detectable viral antigen between the epidermal downgrowths and by day 30 no viral antigen was visible in any of the sheep.

A)



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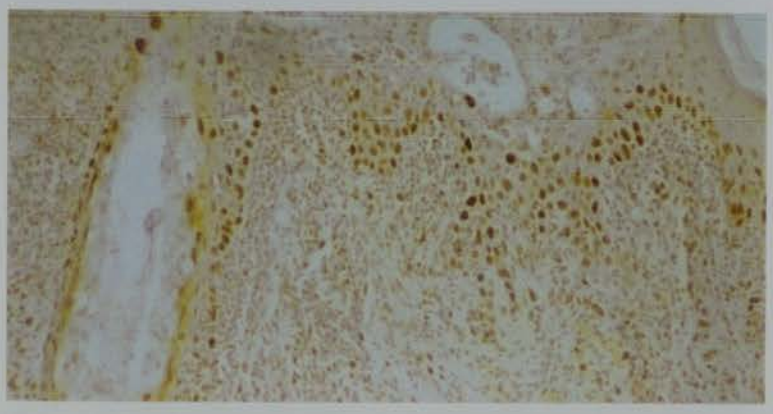

C)

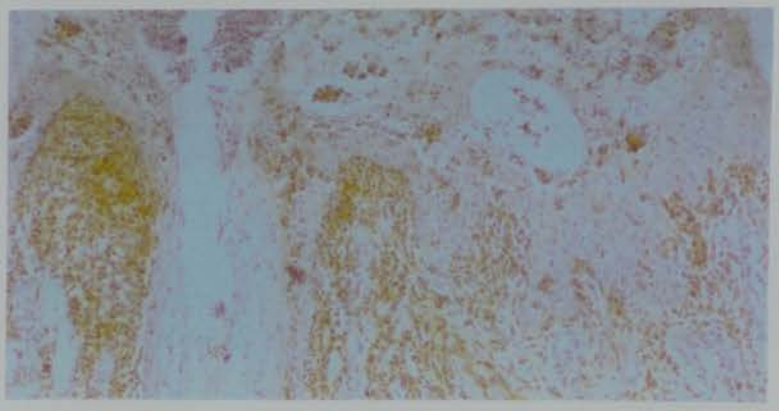

**Figure 3.10** *Serial sections of ovine skin 170h post-primary infection with orf virus showing* **A)** *the distribution of orf viral antigen (anti-orf antibody 2E5) at the very outer layers of the degenerating epidermis that lie directly above* **B)** *the areas of epidermal proliferation (PCNA positive) and* **C)** *the factor XIIIa$^+$ dendritic cells that form a close association with areas of epidermal proliferation. Immunoperoxidase* **X 112**

*Secondary orf virus infection*

Like the primary response, viral antigen was not seen at 48h. At 96h viral antigen was detected within the ballooning degenerated cells of the infected epidermis underlying the scab. Little proliferative activity in the underlying epidermal cells was observed. By 170h post-infection no viral antigen was found.

## 3.3. DISCUSSION

Our studies show that within normal ovine skin the MHC class II$^+$ dendritic cells comprise at least four sub-populations of dendritic cells; one is found exclusively within the epidermis- the LC and is Ache$^+$ and CD1$^+$. The Ache$^-$ dermal dendritic cells described by Jenkinson *et al.*, (1991) constitute three sub-populations of dendritic cells, CD1$^+$ dendritic cells (that represent about 45% of all dermal dendritic cells) and CD1$^-$ dendritic cells of which a proportion (approximately 30%) are positive for the marker factor XIIIa.

The MHC class II$^+$ dendritic cells that gather in response to scarification and orf virus infection (primary and secondary) were found to be a population of CD1$^-$ cells. In all three of the groups the dendritic cell accumulation was similar at 48h, confirming the work by Jenkinson *et al.*, (1991) which showed that the initial dendritic cell response in experimentally infected animals is due to the procedure of scarifying the skin. From 48h onwards the increase in dendritic cells is in response to viral replication and the subsequent degeneration of infected epidermis, shown to contain viral antigen. Meanwhile the numbers of dendritic cells in scarified skin return to normal. A larger and more prolonged accumulation of dendritic cells is observed in a primary orf virus infection compared with a secondary challenge infection. This correlates with the persistence of the virus in a primary infection, where viral antigen is still detectable 16 days post-infection. In contrast, the secondary infection resolves much faster, with the clearance of viral antigen from the lesion by 170h post-infection. It would have been interesting to study the response to inactivated orf virus to confirm the importance of the replicating virus in cellular recruitment.

Factor XIIIa$^+$ dendritic cells appear to form part of the dendritic cell response to epidermal injury; they are observed 48h after scarification alone and may have a role in the initial healing process of the skin. Evidence to support this putative role of factor XIIIa$^+$ dendritic cells comes from recent studies in humans which show that factor XIIIa is found regularly in the early stages of repairing lesions, most clearly in

evolving scars (Penneys *et al.*, 1990). Factor XIIIa is a transglutaminase involved in the final stages of clot formation (Mosher *et al.*, 1980) and one theory suggests that XIIIa is stored in the cells for extracellular release after local injury, crosslinking the fibrin clot to structural protein and helping to seal the wound (Penneys *et al.*, 1990). This theory of factor XIIIa deposition at the scar could explain the diffuse non cell-associated staining often observed within the scab and at the edges of the lesion and may also be the reason for the transient expression by the cells. Factor XIIIa$^+$ dendritic cells were present in much higher numbers and persisted for much longer in the primary orf lesion than in the scarified skin and secondary orf lesion. Factor XIIIa$^+$ dendritic cells formed a considerable part of the dendritic cell network in the primary orf lesion, persisting until the viral antigen was cleared. This may have been due to the more extensive nature of the lesion, or these cells may have a role other than in wound healing.

Recently, human factor XIIIa$^+$ dendritic cells have been identified as coexpressing intracellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen (LFA-1). The presence of these immunological markers and the distinctive distribution of these cells, with the highest concentration being in the skin and mucosal tissues, has led to the suggestion that they play a central role in skin immune responses (Derrick *et al.*, 1993). Other studies have reported possible phagocytic and antigen processing functions of factor XIIIa$^+$ dendritic cells (Foucar and Foucar 1990). Factor XIIIa$^+$ dendritic cells have been demonstrated in psoriatic skin lesions as being potent producers of tumour necrosis factor -$\alpha$ (TNF$\alpha$) (Nickoloff *et al.*, 1991), which is implicated in the increased growth rate of the keratinocytes in the lesions. It was hypothesised that the ability of the dermal dendritic cells to produce TNF$\alpha$ could increase lymphocyte trafficking through the skin by inducing IL-8 and ICAM-1 production by keratinocytes (Nickoloff *et al.*, 1991). Thus a possible function of the cells could be the attraction of neutrophils and T lymphocytes into the skin in response to epidermal damage or viral replication. High numbers of factor XIIIa$^+$ dendritic cells are seen in the verrucous skin lesions of HIV-1$^+$ patients caused by secondary infections with DNA viruses, such as varicella zoster virus, herpes simplex

virus and molluscum contagiosum (Smith *et al.*, 1992). The ability of factor XIIIa$^+$ dendritic cells to influence keratinocyte growth via TNF$\alpha$ production could contribute to the process of healing damaged skin. Interestingly, factor XIIIa$^+$ dendritic cells were most numerous adjacent to the epidermal downgrowths which are characteristic of primary but not secondary orf lesions. A similar association of factor XIIIa$^+$ dendritic cells with epidermal cell proliferation is observed in psoriasis. As the secondary orf lesion heals well without the presence of these cells, the high numbers of factor XIIIa$^+$ dendritic cells that accumulate in a primary orf lesion may have an immunological role, possibly reflecting fundamental differences in the primary and secondary immune response to orf virus infection and contribute to the epidermal proliferation observed.

CD1$^+$ dendritic cells do not form part of the dendritic cell network in the responses to scarification, primary or secondary orf virus infections. The number of CD1$^+$ dendritic cells in the dermis decreased with the onset and spread of virus infection, the depletion being most marked at the peak of a primary infection. The function of the family of CD1 molecules is poorly defined, but they share a resemblance to MHC class I molecules (Terhorst *et al.*, 1981 and Knowles *et al.*, 1982) and have been implicated as playing a role in cell mediated endocytosis and antigen presentation (Hanau *et al.*, 1987 and Kapsenberg *et al.*, 1990). CD1$^+$ dendritic cells have been characterised in ovine afferent lymph (Budjoso *et al.*, 1989) and are postulated to act as accessory cells for T lymphocytes. It could be hypothesised that the disappearance of CD1$^+$ dendritic cells from the skin reflects their migration to the draining lymph node via the afferent lymphatics following orf virus infection, to present viral antigen to the T lymphocytes. It is unknown, however, whether the antigens CD1 and factor XIIIa can be influenced by the focal effects of inductive cytokines. The differences in the level of expression of factor XIIIa between primary and secondary responses could be explained by differences in the local cytokine profiles. The depletion of CD1 from the surrounding dermis may occur as a direct result of orf virus replication and the induction or suppression of local cytokines which may down regulate CD1

expression. The levels of CD1 return to normal only when the infection is well into regression.

Cerio *et al.*, (1989) studied dermal dendritic cells that expressed factor XIIIa and concluded that they were bone marrow derived, distinct from LCs and shared some common epitopes with mononuclear phagocytes. The relationship of the dendritic cell with the monocyte-macrophage lineage is still under debate. The lack of staining of the MHC class II$^+$ dendritic cells with a panel of monoclonal antibodies which recognise markers on macrophages, confirms that these cells are not activated macrophages expressing MHC class II, but still leaves their origin unknown. Factor XIIIa is also expressed by monocytes and is retained during their differentiation into macrophages. It is possible that the factor XIIIa$^+$ dendritic cells observed in a primary orf virus infection represent an immature population of dendritic cells, the subsequent maturation and differentiation of which is dependant upon an array of cytokines produced locally. The differences in the level of expression of factor XIIIa between the two populations of CD1$^-$ dendritic cells below the primary and secondary lesions may reflect differences in the cytokine profiles present.

As the accumulation of cells in response to scarification and secondary orf virus infection occurs in the absence of any proliferative activity, it is unlikely that the small population of MHC class II$^+$, CD1$^-$ dendritic cells present in normal ovine skin proliferate locally to form the dense network of dendritic cells. High proliferative activity is observed within the dermal influx in primary orf virus infections. A large proportion of this may be due to the local proliferation of B/T cells that is not observed in the secondary response where memory cells come into play. Dual staining revealed that a few of these proliferating cells are MHC class II$^+$ dendritic cells. It seems more likely that the large accumulation within the dermis arises from an influx of cells from the blood in response to changes in surface markers of the endothelial cells lining the blood vessels of the skin. This may represent a positive selection of circulating dendritic cells or a population of precursor cells that go on to develop their dendritic characteristics after infiltrating the tissues. This could be investigated by the

application of antibodies to adhesion molecules such as ICAM-1 to sections of orf infected skin.

The proliferation of epidermal keratinocytes appears to be a pre-requisite for orf virus infection. The initial procedure of scarifying the skin permits the access of orf virus into the skin, where it replicates in the regenerating keratinocytes involved in the healing of the abrasion. The absence of orf viral antigen within the first 48h post-infection is known as the eclipse phase and it is hypothesised that the virus resides within the replicating cells but requires them to reach a certain stage in their cell cycle before it itself, can replicate. This pattern of proliferation followed by the appearance of viral antigen is much more prolonged in a primary infection. In the primary orf lesion the extensive epidermal proliferation that occurs results in the formation of epidermal downgrowths, which initially are PCNA positive but lack orf viral antigen. A few days later, however, the reverse is true, they lose their PCNA reactivity and are shown to contain viral antigen. The less proliferative nature of the secondary orf lesion may restrict the spread of the virus and result in a much milder lesion.

In summary, the dendritic cells accumulating in orf virus infected skin share the phenotype of the population of $CD1^-$ dermal dendritic cells found in normal ovine skin. A proportion of these cells express the marker factor XIIIa, the number varying considerably between primary and secondary responses. Detailed functional studies will be required to determine whether factor XIIIa and CD1 are inducible on these cells and if the expression of the different markers reflects functional differences, such as antigen presentation or cytokine production, between the different subpopulations of dendritic cells. The information gained from these studies should then provide an insight into the possible origin and role of dendritic cells at the site of an orf virus infection.

# CHAPTER 4

## ESTABLISHMENT OF KERATINOCYTE CULTURES FOR *IN VITRO* STUDIES OF ORF VIRUS REPLICATION

## 4.1. INTRODUCTION

Detailed *in vivo* studies of the orf lesion located orf viral antigen within the newly regenerating epidermal keratinocytes, identifying the keratinocyte as the site of orf virus replication (Jenkinson *et al.*, 1990a,c). Whilst orf virus will replicate *in vitro* in a variety of different cell types (Robinson and Balassu 1981), there have been no reports of orf virus growth in keratinocytes. It became apparent from the many *in vitro* studies of orf virus, that the onset of cytopathic effect (CPE) was variable ranging from 2h-48h post-infection. The factors involved in this variation were considered to include the titre of infectivity, the age of the cultures, with orf virus reportedly replicating to higher titre in young rapidly dividing cells, and the type and batch of cells used (Nagington *et al.*, 1968). These studies highlight the need to investigate, *in vitro*, the kinetics of orf virus replication in the target cell of the natural infection - the keratinocyte.

Epidermal keratinocytes can grow in culture to form a stratified squamous epithelium (Holbrook and Hennings, 1983). These cultures contain a dividing as well as a terminally differentiating population and undergo surface desquamation. The establishment of such keratinocyte cultures is, however difficult due to the problem of fibroblast contamination which occurs as a direct result of using skin samples obtained from circumcision, surgical waste or by biopsy as a source of cells. This problem was initially overcome by Rheinwald and Green (1975) through the use of a feeder layer of lethally irradiated 3T3 fibroblasts which support the growth of keratinocytes and prevent fibroblastic overgrowth.

A variety of different methods for growing keratinocytes in culture have since been developed, most of which are based on the technique of Rheinwald and Green (1975). The cultivation of keratinocytes, although now routine, is nevertheless complex. The desire to produce a more normal epidermis *in vitro* for experimental purposes has led to the development of substrates of collagenous gels, including extracellular matrix components, or constructs of dermal equivalents (Bell *et al.*, 1983, Hansborough *et*

*al.*, 1989, Yannas *et al.*, 1989). Other studies have favoured the use of conditional media containing added growth factors such as epidermal growth factor (Rheinwald and Green, 1977) and cholera toxin (Green *et al.*, 1978), which encourage keratinocyte growth in the absence of a feeder layer.

A unique method for culturing keratinocytes was established by Weterings *et al.*, (1981) where the follicular epithelium of plucked hair follicles was used as a source of cells. The plucked hairs were explanted and colonies of keratinocytes formed from the follicular epthelium surrounding the hair. A number of workers went on to develop this technique using a variety of substrates, such as bovine lens capsules with varied success (Wells, 1982; Weterings *et al.*, 1981; Limat and Noser, 1986; Wells and Sieber, 1985; Sieber and Wells, 1986; Stark *et al.*, 1986; Imke *et al.*, 1989; Sieber and Hopewell, 1990), the major drawback being the limited cell numbers that could be obtained. The absence of dermal components from the plucked hair follicle reduces the risk of fibroblast contamination, therefore providing a relatively pure source of keratinocytes. This system has been described for plucked sheep hairs (V.K. Sieber, personal communication) and would appear to be a simple technique to obtain ovine keratinocytes that is less invasive and more selective than the alternative method of biopsying skin as a source of cells.

The aim of this study was to establish pure cultures of ovine keratinocytes from plucked sheep hairs, infect them with orf virus and determine the kinetics of viral replication and development of CPE. This preliminary study provides the necessary information about the time course of infection for the subsequent studies of cytokine production by orf virus infected keratinocytes described in chapter 5.

## 4.2. RESULTS

The details of the materials and methods used in this chapter are described in chapter 2.

### 4.2.1. Establishment of ovine keratinocyte cultures

*i). Primary cultures*

Several attempts to grow keratinocytes from plucked hairs in some of the commercially available keratinocyte media were unsuccessful. The first successful primary cultures from plucked hairs were achieved in the keratinocyte medium described by Wells and Sieber (1985) with additional growth factors; hydrocortisone (0.4μg/ml) and epidermal growth factor (EGF, 10ng/ml). These were used at the optimal concentrations established for human keratinocyte cultures (Limat *et al.*, 1986, Lin *et al.*, 1988).

After approximately 3-5 days of culture, the first signs of keratinocyte growth were visible. Small outgrowths of balloon-like cells could be observed, originating from the outer root sheath of the plucked hair. Cells then appeared to radiate outwards from the attached hair follicle to form a small colony of cells with polygonal morphology (Figure 4.1A). Only a small percentage of the attached hair follicles of each flask gave rise to a colony of growing keratinocytes (<30%). The successfully established colonies increased slowly in size reaching approximately 1cm in diameter after 14-20 days incubation. Figure 4.1B shows a 14 day old keratinocyte colony. Variations in the cell morphology were observed across the radius of the outgrowth. The cells at the boundary of the colony were a single cell thick and had a flat shape with some cellular processes reaching out into the surrounding area (similar to the cells of the early outgrowth observed in Figure 4.1A). Towards the centre of the colony, closest to the follicular epithelium, the cells were densely packed, stratified and several layers in thickness. Occasionally the layers of keratinocytes became so thick that they formed regions of opalescent, raised areas (domes), most often observed towards the

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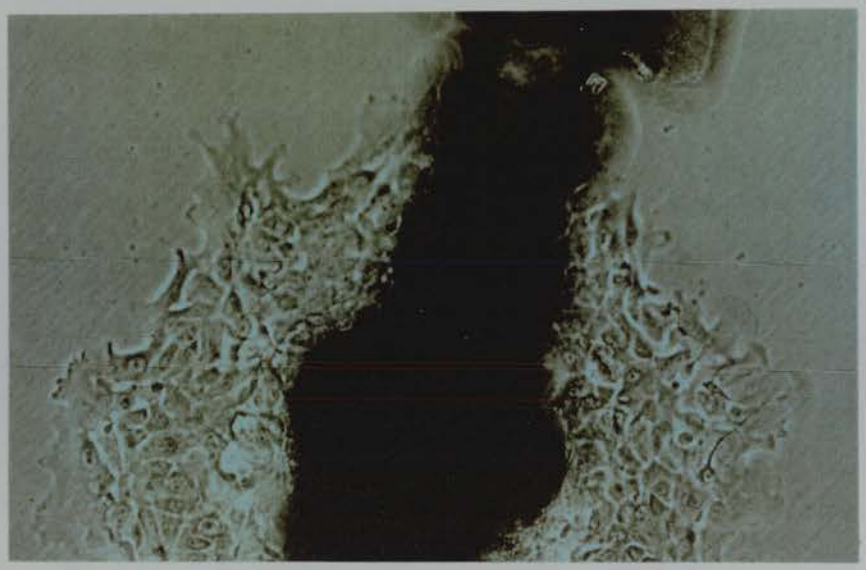


B)

**Figure 4.1** *Outgrowth of keratinocytes from the follicular epithelium of a plucked sheep hair following* **A)** *5 days incubation and* **B)** *14 days incubation. Phase contrast* **X 112**

centre of the outgrowth. After 14 to 20 days in culture the cells were trypsinised for the first time. At this stage the cell outgrowths did not cover the entire surface of the culture flask, if they were left longer however, the colonies increased in thickness, but not in diameter. The multilayered colonies of cells were often difficult to detach from the culture flasks. Detachment was aided by a longer incubation in trypsin combined with gently tapping the sides of the flasks. After the first trypsinisation the cells were returned to the original flasks to encourage cell attachment. They were then grown in medium containing no supplementary growth factors. Usually within 3-4 days of their first passage the cells formed a uniform monolayer (Figure 4.2).

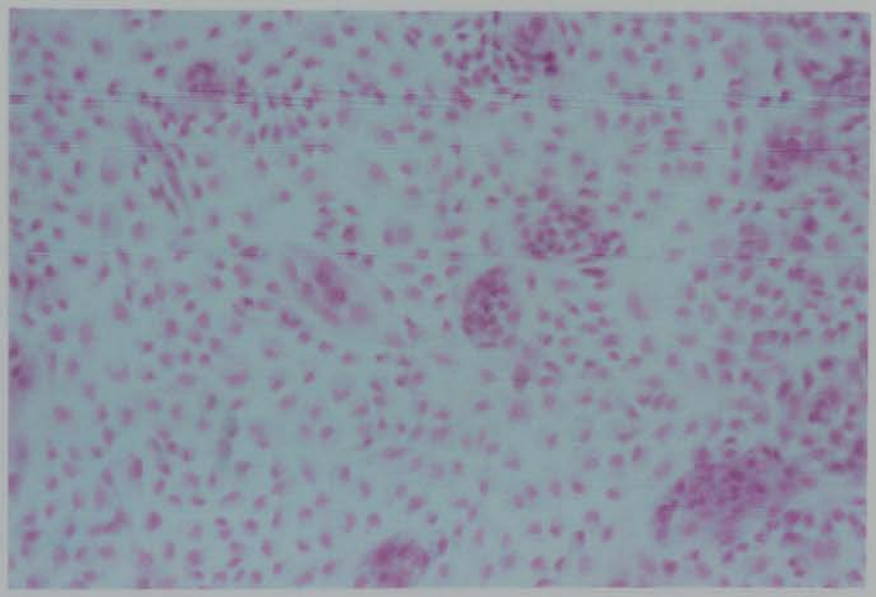


**Figure 4.2** *Monolayer of keratinocytes at first passage. Leishman's* **X 112**

### *ii). Subculture*

The keratinocytes grew well in the medium described and a single flask ($25cm^2$) of keratinocytes obtained after the first passage could be split 1 to 2, for repeated passages, 5 or more times to give 32 flasks ($25cm^2$) of cells in approximately 4 to 5 weeks. Attempts to culture the cells beyond a sixth passage were unsuccessful.

Keratinocytes collected and stored after the second trypsinisation were successfully recovered from liquid nitrogen and could be passaged as described.

### 4.2.2. Immunohistochemical characterisation of keratinocytes

Due to the virtual absence of dermal components in the plucked hair follicle, overgrowth of the cultures by fibroblasts was rarely encountered. The characteristic morphology and slow growth rate of the keratinocytes was a good indication of the purity of the cultures. Any fibroblast contamination was clearly apparent due to their fast growth rate and fibroblastic morphology. The purity of the cultures were, however, evaluated by immunoperoxidase with antibodies against keratin polypeptides which are synthesised in the epidermis and cultured keratinocytes. The anti-keratin antibody was shown to stain the keratinocytes surrounding a hair follicle in a section of normal ovine skin (Figure 4.3).

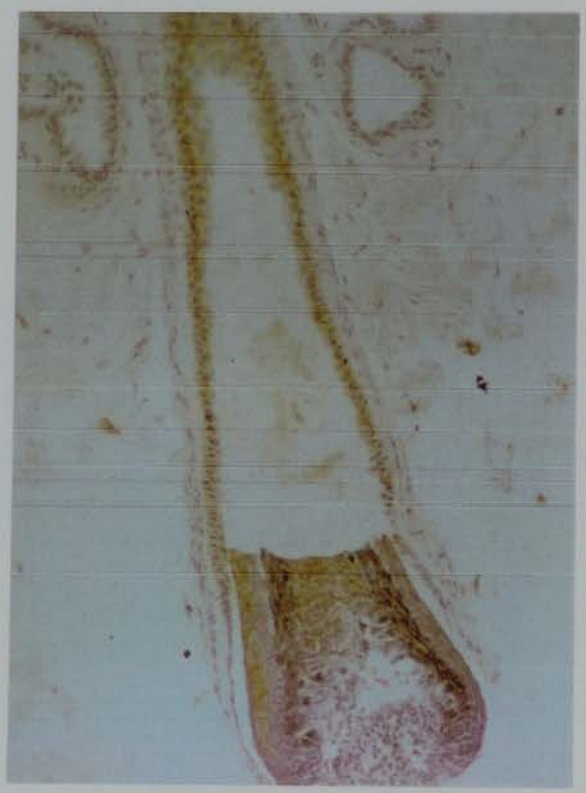


**Figure 4.3** *Paraffin section (10μm thick) of normal skin showing anti-keratin postive keratinocytes lining a hair follicle. Immunoperoxidase* **X 112**

An antibody was also included that reacts with vimentin; a marker for mesenchymal cells (including fibroblastic cells). Monolayers of foetal lamb (FL) skin cells which should contain both populations of keratinocytes and fibroblasts were also stained with the two antibodies.

*i). Anti-keratin*

Approximately 99% of the keratinocyte monolayers were positive for keratin (Figure 4.4A). The antibody could be seen to stain intensely tonofilaments of keratin that were present within the cytoplasm of the cells (Figure 4.4B). The population of cells within the FL skin cultures was observed to be largely fibroblastic and these cells did not stain with anti-keratin. However, small islands of smaller, fatter cells could be observed in the cultures that did appear to stain with anti-keratin and these cells were assumed to be keratinocytes (Figure 4.5A).

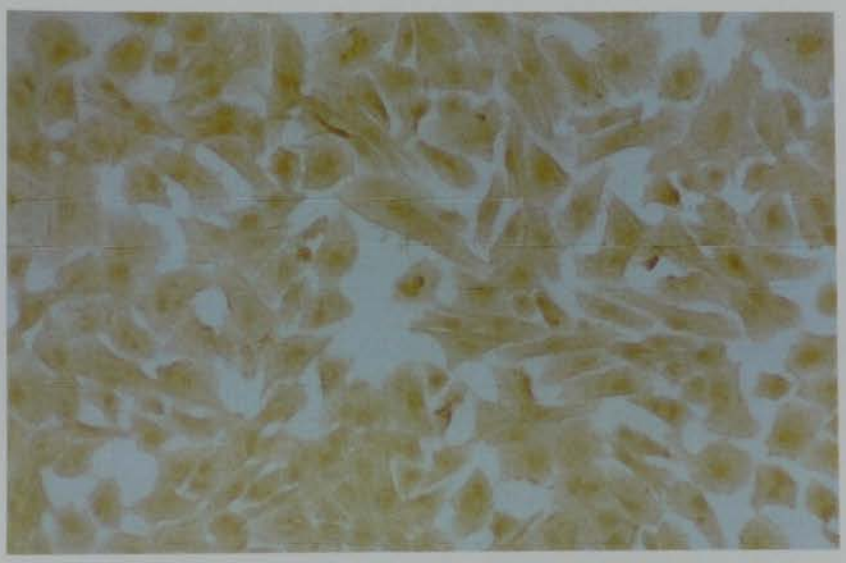
*ii). Anti-Vimentin*

The keratinocyte cultures were negative for vimentin (not shown). The majority of the cells in the FL skin cultures were, however, intensely positive for vimentin (Figure 4.5 B). The smaller islands of cells that were observed to stain with anti-keratin did not stain with anti-vimentin, again indicating their keratinocyte nature.

### 4.2.3. Orf virus infection of keratinocytes

Initial studies of orf virus that were carried out at the Moredun Institute used a virus isolate derived from natural cases to experimentally infect sheep for *in vivo* studies. This scab virus isolate was adapted to tissue culture for *in vitro* studies and designated orf 11 (Mckeever, 1986). Orf 11 does not, however, produce experimental orf lesions *in vivo* (Dr H.W.Reid, personal communication). More recently, experimental work has favoured the use of a commercially available tissue culture adapted strain of orf virus, Scabby Mouth (see chapters 2 and 3). This strain of orf virus represents a purer inoculum than the undefined components of the scab virus and can be used in both the *in vivo* work, as it will produce lesions experimentally, and in the *in vitro* studies. In this study both strains of tissue culture adapted orf

A)



B)

**Figure 4.4** *Monolayer of anti-keratin positive keratinocytes at* **A)** *low magnification* **X 112** *and* **B)** *high magnification.* **X 560**. *Immunoperoxidase*

A)

B)

**Figure 4.5** *Monolayers of FL skin cells stained with* **A)** *anti-keratin, showing a single positive cell and* **B)** *anti-vimentin, where the majority of cells are positive. Immunoperoxidase* **X 560**

(orf 11) and Scabby Mouth were included and their ability to replicate within ovine keratinocytes determined.

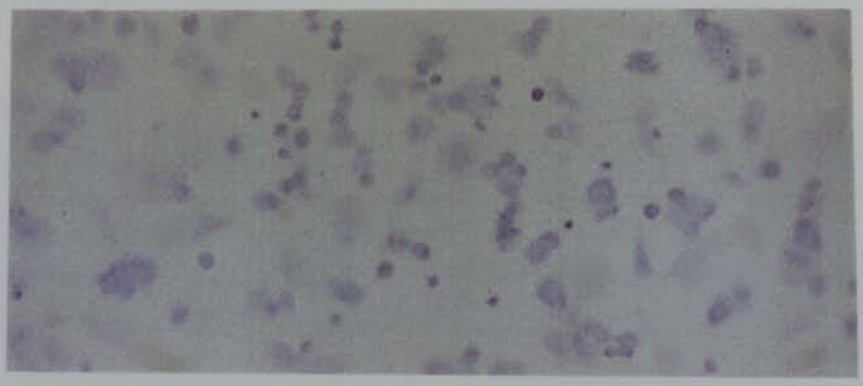
### *i). Titration of orf virus in keratinocytes*

Keratinocyte monolayers grown on plastic coverslips in 48 well plates were infected with tenfold dilutions of Scabby Mouth or orf 11 and the plates examined for viral cytopathic effect (CPE) at 12h, 24h and then daily until day 4. The final titre was calculated on day 4 following the method described by Karber (1931). Fixed monolayers of infected cells at 12h, 24h and 48h were stained with Leishman's and photographed.

#### *Cytopathic effect (CPE)*

The coverslips infected with the $10^{-1}$ dilutions of virus were fixed and stained with Leishman's at 12h, 24h, and 48h to observe the CPE of both Scabby Mouth and orf 11. The Scabby Mouth virus preparation that was used gave a rapid CPE that caused cell death and marked disruption to the monolayer by 12h post-infection (Figure 4.6A). This CPE was not observed for the orf 11 infected cells, as at 12h the monolayer was intact and the only indication of infection was the presence of some small pyknotic cells (Figure 4.6B) that were not observed in the uninfected control cells (Figure 4.6C). At 24h post-infection virtually 99% of the cell monolayer was disrupted by Scabby Mouth; all the cells that were still attached were rounded (Figure 4.7A). A visible CPE was now detected for orf 11 infected cells, with some cell rounding and the appearance of patches in the monolayer was observed (Figure 4.7B). By 48h post-infection with Scabby Mouth very few cells were left attached to the coverslip. At this timepoint a dramatic CPE was observed for the orf 11 infected cells (Figure 4.8A and B), which was only now comparable to the CPE observed for Scabby Mouth at 12h post-infection.

A)



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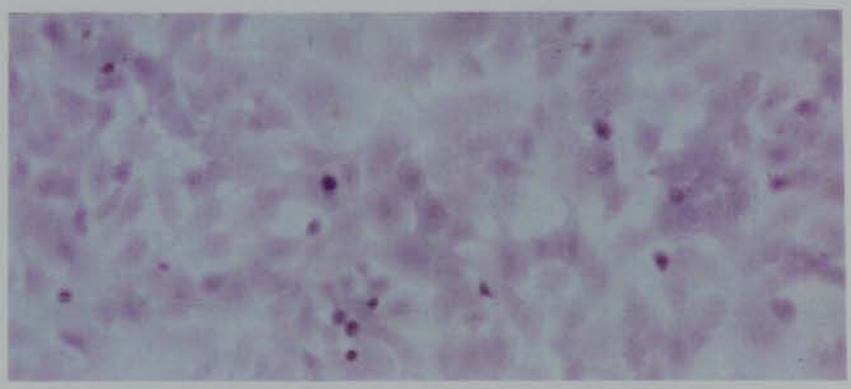


C)

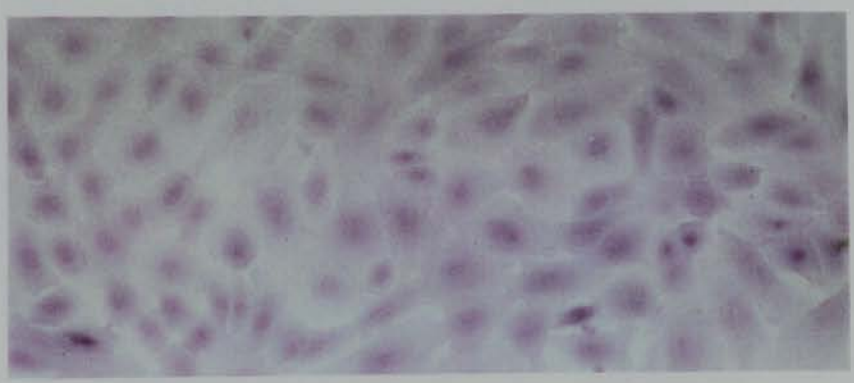
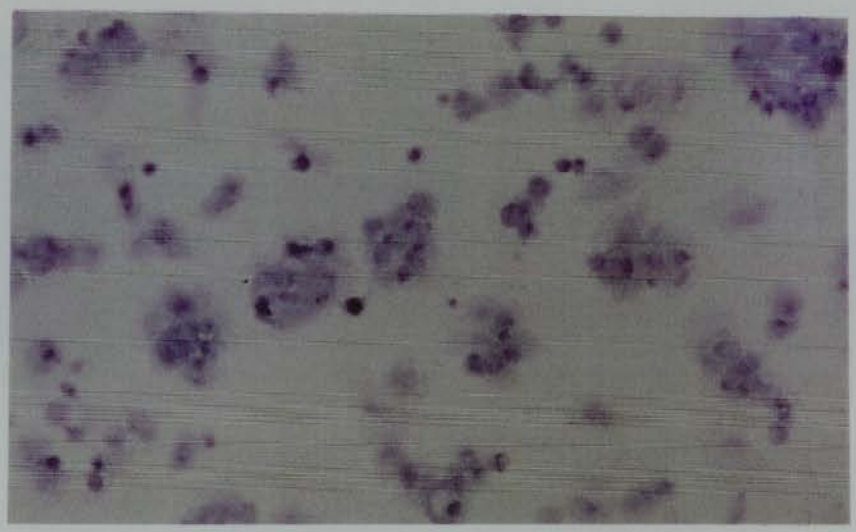


**Figure 4.6** *Monolayers of keratinocytes at 12h post-infection with* **A)** *Scabby Mouth virus and* **B)** *orf 11 virus and* **C)** *uninfected control cells . Leishman's stain* **X 112**

A)



B)

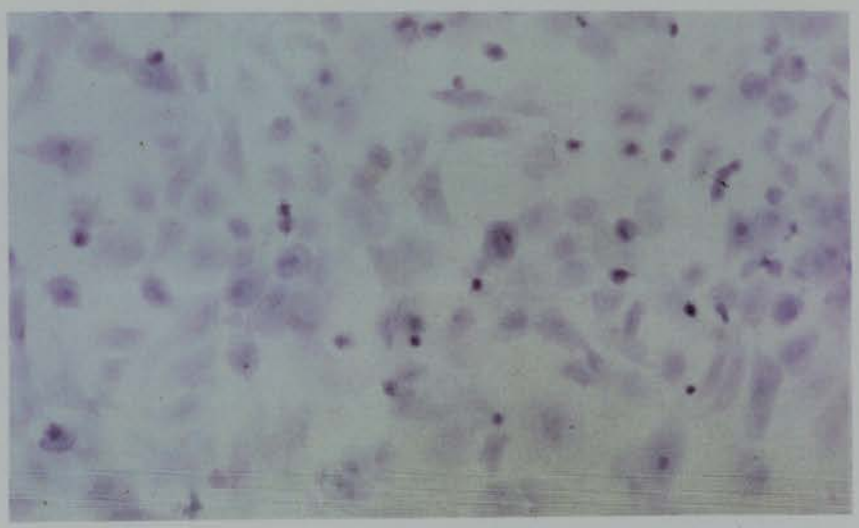
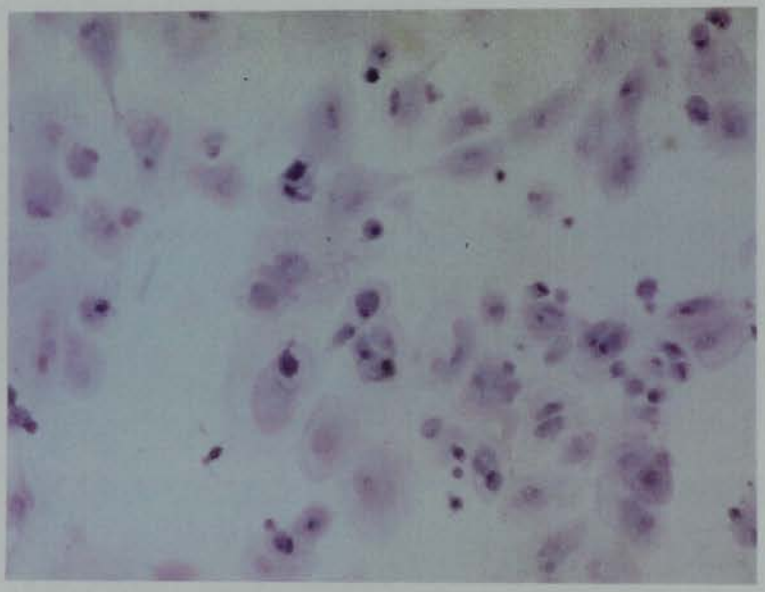


**Figure 4.7** *Monolayers of keratinocytes at 24h post-infection with* **A)** *Scabby Mouth virus and* **B)** *orf 11 virus. Leishman's stain* **X 112**

A)



B)

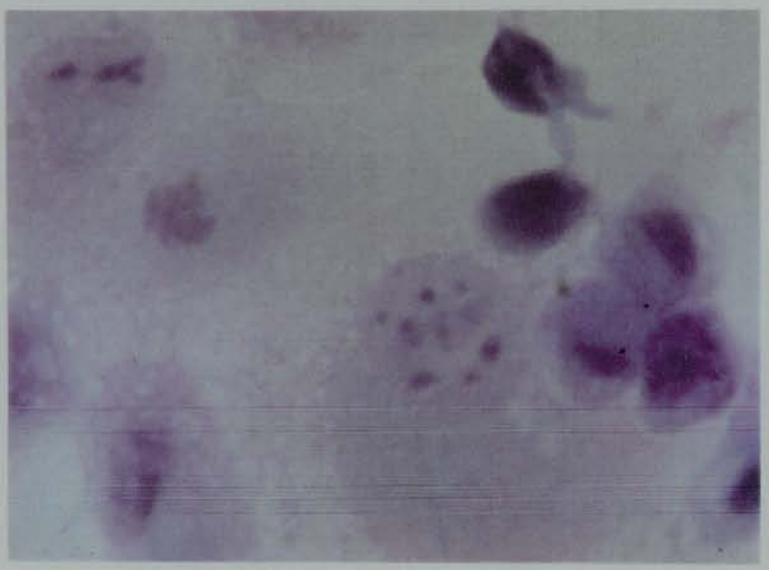


**Figure 4.8** *Monolayers of keratinocytes at 48h post-infection with orf 11 virus* **A)** *at low magnification* **X 112** *and* **B)** *at high magnification* **X 560**. *Leishman's stain.*

*Viral titres*

The total proportion of positive wells (i.e wells that showed CPE) of each dilution was summed (see Table 4.1) and used to calculate the 50% tissue culture infectious dose ($TCID_{50}$) of the stock virus (Karber, 1931).

**Table 4.1** *Orf virus titration readings showing the proportion of positive wells for each dilution.*

| Viral dilutions | Proportion of positive wells | |
|---|---|---|
| | Scabby Mouth | Orf 11 |
| $10^{-1}$ | 6/6= 1 | 6/6= 1 |
| $10^{-2}$ | 6/6= 1 | 6/6= 1 |
| $10^{-3}$ | 6/6= 1 | 6/6= 1 |
| $10^{-4}$ | 6/6= 1 | 6/6= 1 |
| $10^{-5}$ | 3/6= 0.5 | 3/6= 0.5 |
| $10^{-6}$ | 0/6= 0 | 0/6= 0 |

The $TCID_{50}$ calculated from the above readings was found to be $10^6$/ml for both Scabby Mouth and orf 11. Therefore, despite the accelerated CPE of Scabby Mouth, both virus preparations contained the same infectivity.

*ii). Detection of viral antigen in infected keratinocytes*

Monolayers of keratinocytes infected with Scabby Mouth or orf 11 preparations were examined for the presence of viral antigen at 12h, 24h and 48h post-infection by immunofluorescence and immunoperoxidase.

*Immunofluoresence*

Using orf hyperimmune serum as a first step antibody (see chapter 2 section 2.2.2 (iv)) orf viral antigen was detected at 12h post-infection in the cytoplasm of single or small groups of infected cells dotted across the monolayer for both strains of viruses

(Figure 4.9A). No difference was observed between the two strains of viruses despite the accelerated CPE of Scabby Mouth. By 24h, groups of fluorescing cells could be observed, which for orf 11 were beginning to round up and detach from the culture vessel (Figure 4.9B). The distribution of viral antigen in the monolayer was similar for Scabby Mouth, although the CPE was well advanced. At 48h post-infection clumps of brightly fluorescing rounded cells were observed for orf 11 (Figure 4.9C) and the cells infected with Scabby Mouth, that were still attached, were also brightly positive.

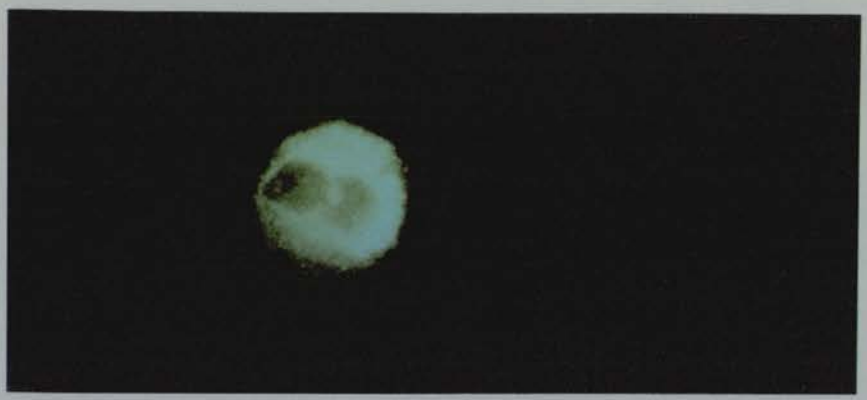
***Immunoperoxidase***

The anti-orf antibody 5E2, that was used to detect orf viral antigen in the *in vivo* orf lesions described in chapter 3, also recognised viral antigen in the infected cultures of cells. No difference between the appearance of viral antigen was observed for the two strains of virus. At 12h post-infection, single cells in the monolayer could be seen to be faintly positive for orf virus antigen (Figure 4.10A). By 24h, a larger number of cells in the monolayer were positive and the viral antigen was located in large inclusion bodies within the cytoplasm of the cell. By 48h the cells were rounded and densely packed with viral antigen (Figure 4.10B).

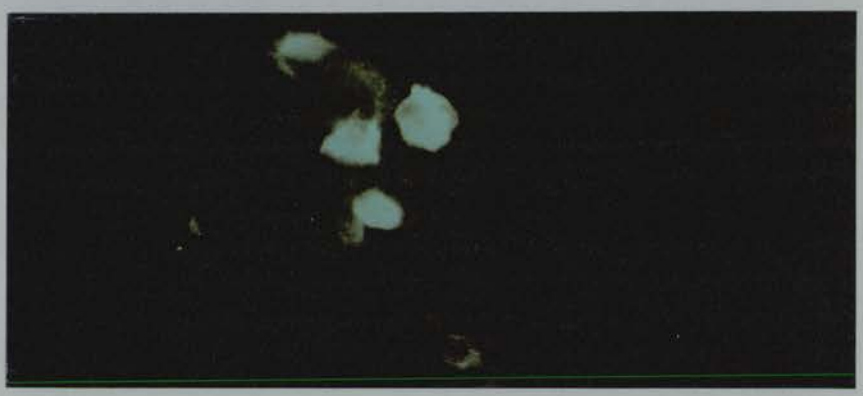
### *iii). Viral growth curves*

One step growth curves for Scabby Mouth and orf 11 were carried out to determine the kinetics of orf virus replication in keratinocytes. A series of keratinocyte monolayers were infected with either Scabby Mouth or orf 11 and incubated at 37°C. At various stages during the growth cycle, flasks were removed from incubation at 37°C and viral replication arrested by freezing the cells and supernatants separately at -70°C. Infectivity in both the cells and supernatants for each time point was then determined by titrating the samples on pre-formed monolayers as described above for the stock virus.

A)



B)



C)

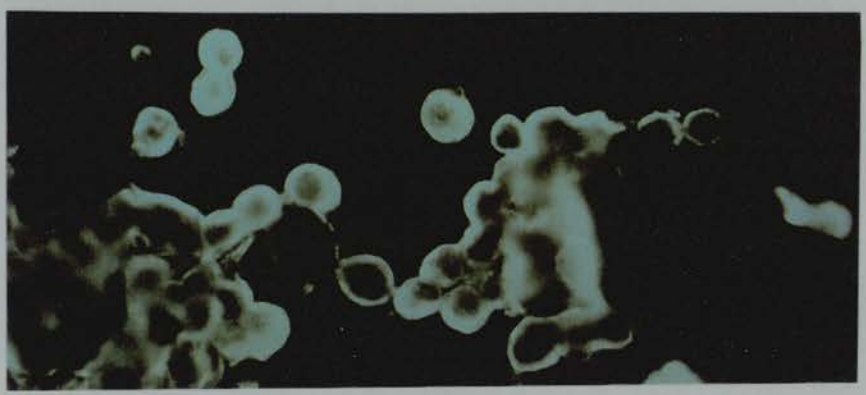


**Figure 4.9** *Immunofluorescent staining of orf virus infected keratinocytes at* **A)** *12h post-infection* **B)** *24h post-infection and* **C)** *48h post-infection.* **X 448**

A)

B)

**Figure 4.10** *Immunoperoxidase staining of orf virus infected keratinocytes with the antibody 5E2 at* **A)** *12h post-infection and* **B)** *48h post-infection* **X 560.**

The $TCID_{50}$ of the cell and supernatant samples for Scabby Mouth and orf 11 at each timepoint post-infection were calculated as described before and the results plotted (Figures 4.11A and 4.11B).

Overall, the kinetics of viral replication for both strains of virus were similar. Initially, in the first 8h post-infection an eclipse phase was observed, where the number of infectious virus particles detected decreased for both cells and supernatants. This reflects the penetration and uncoating of the virus in the cytoplasm of the cell (Onwuka, 1989). From 8h onwards, orf virus replication occurred with an increase in the number of infectious progeny detected in both the cells and supernatants. Early in infection orf virus appears to remain cell-associated; infectious virus in the cells and supernatants increased in parallel until about 48h post-infection when the amount of virus released into the supernatants was higher than the cell-associated virus. By 72h the cell-associated virus decreased as the dead cells can no longer support viral replication and the highest number of infectious virus particles were detected in the supernatants.

There was however, a slight difference between the growth curves of the two strains of orf virus. Between 12h and 24h, the number of infectious particles released by Scabby Mouth increased dramatically before levelling off between 24h and 48h. In contrast, orf 11 was slow to release infectious virus, with an increase first detected between 24h and 48h. This difference may be associated with the accelerated CPE observed for Scabby Mouth. At 72h orf 11-infected cultures had higher titres in the supernatants than detected with Scabby Mouth indicating that although orf 11 is slower to initiate the release of infectious virus and to cause a visible CPE it may be the more efficient of the two strains of virus at replicating.

### *iv). Incorporation of $^{35}S$ methionine by keratinocytes.*

This study was carried out to determine the effect of orf virus infection on keratinocyte protein synthesis. Monolayers of keratinocytes grown to 80% confluence were infected with orf virus and at two timepoints post-infection (early = 2h and late

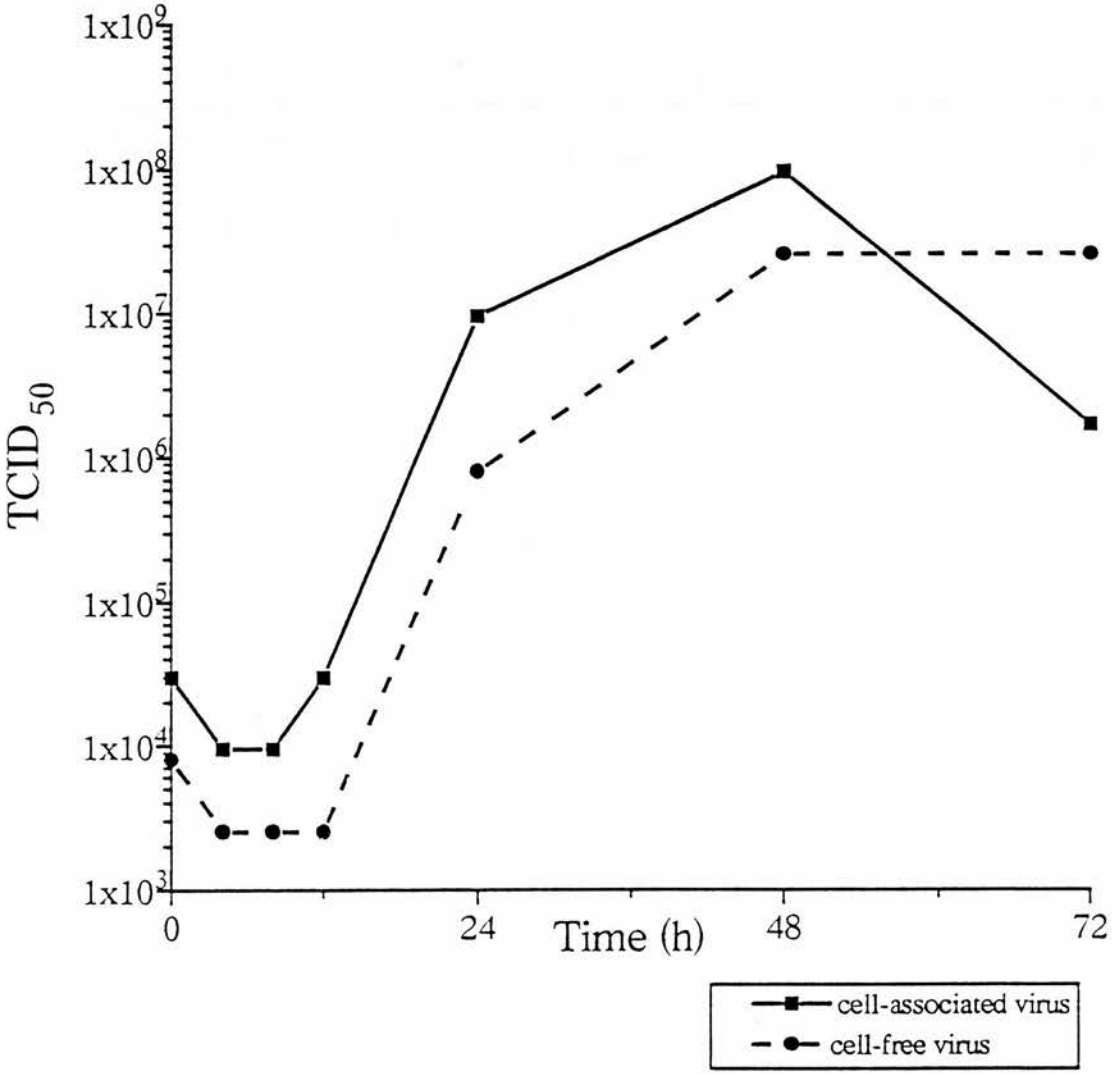



**Figure 4.11A** *Orf virus replication (Scabby Mouth) in ovine keratinocytes. 25cm² monolayer cultures were infected with a MOI of 0.5 and the $TCID_{50}$ of cell-free and cell-associated virus determined at timepoints post-infection. The $TCID_{50}$ for each timepoint is expressed as total infectivity present.*

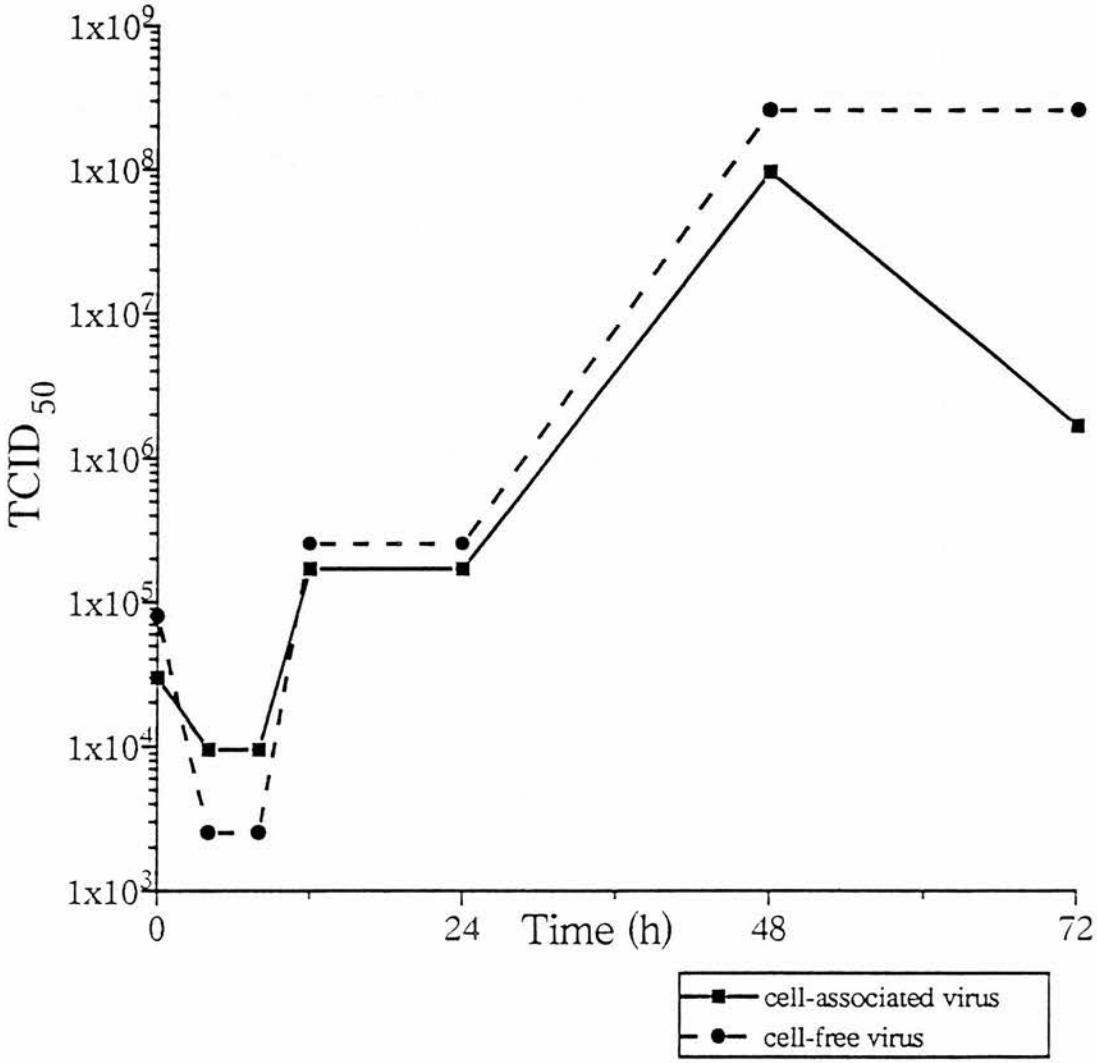



**Figure 4.11B** *Orf virus replication (orf 11) in ovine keratinocytes. Monolayer cultures were infected with a MOI of 0.5 and the $TCID_{50}$ of cell-free and cell-associated virus determined at timepoints post-infection. The $TCID_{50}$ for each timepoint is expressed as total infectivity present.*

= 9h) protein synthesis was measured by the incorporation of $^{35}$ S labelled amino acids. Initial studies analysed both the cell lysates and supernatants from the infected/uninfected cells. However, analysis by SDS page revealed a large number of protein bands from the cells with no obvious qualitative difference of cellular/viral protein expression between infected and uninfected cultures. Thus subsequently only the incorporation of $^{35}$S into proteins that were secreted into the culture supernatants of the infected/uninfected cells was measured. Actinomycin D was included in two of the infected flasks (for early and late collections) in an attempt to inhibit viral protein synthesis and thus to measure the incorporation of $^{35}$S that was entirely due the cellular protein synthesis. Shatkin *et al.*, (1963) had shown that 2μg/ml of actinomycin D could prevent vaccinia virus protein synthesis. However in the present study actinomycin D was used at a concentration of 1μg/ml since at higher concentrations it was found to be toxic to the keratinocytes. The results for the experiment are shown below in Table 4.2 and in Figure 4.12.

**Table 4.2** *$^{35}$S amino acid incorporation into protein secreted into the culture supernatants of infected and uninfected control keratinocytes*

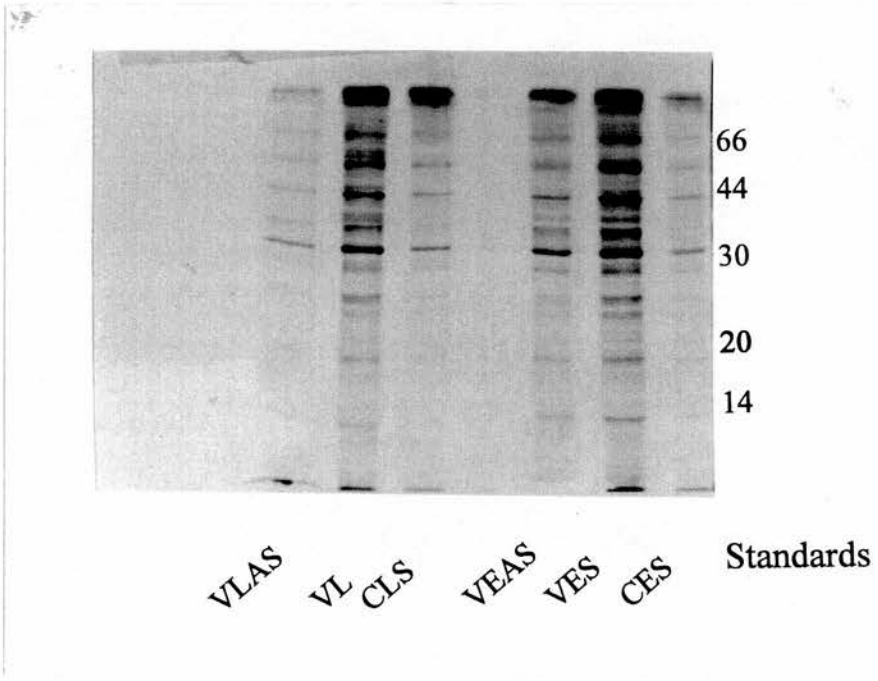
| Cells | TPC* (cpm) | | % Incorporation# | |
|---|---|---|---|---|
| | Early | Late | Early | Late |
| **Control** | 8346 | 10190 | 4.17 | 5.09 |
| **Infected** | 18426 | 15666 | 9.21 | 7.83 |
| **Infected + Act** | 13557 | 12334 | 6.78 | 6.16 |

* TPC represents the trichloracetic acid (TCA) precipitable counts per minute (cpm) of 10μl of a 5ml aliquot of culture medium measured in a scintillation counter with an assumed efficiency of 50% (see chapter 2 for experimental details).

# % Incorporation is the percentage of total $^{35}$S added that was incorporated into secreted protein in each sample.

+ Act = + 1μg/ml of actinomycin D.

Orf virus infection did not result in the shutdown of protein synthesis by keratinocytes. An increase in protein synthesis after orf virus infection was observed at both the early and late timepoints. The protein synthesis measured also includes that of viral proteins as well as cellular proteins. The exact effect of actinomycin D on orf virus protein synthesis or on host cell protein synthesis was not established in this study but it did appear to reduce the overall amino acid incorporation of the infected cells. If actinomycin was indeed inhibiting viral but not host cell protein synthesis, then a comparison with the control would indicate that orf virus infection stimulates cellular protein synthesis. Analysis of the radiolabelled proteins by SDS page revealed that whilst actinomycin D reduced the level of incorporation of the labelled amino acids by the infected cells, a comparison with the untreated, infected cells did not reveal any protein bands that were directly attributable to the virus (Figure 4.12).




CES= control early sample
VES= virus early sample
VEAS= virus early + actinomycin D
CLS= control late sample
VL= virus late sample
VLAS= virus late sample + actinomycin D

**Figure 4.12** *Autoradiograph of early and late protein synthesis of control and orf virus infected keratinocytes.* $^{35}$*S methionine incorporation by cultures of control and orf virus infected keratinocytes + or - actinomycin D was analysed by SDS gel electrophoresis.*

## 4.3. DISCUSSION

Using a modification of the technique described by Wells (1985), cultures of ovine keratinocytes from the follicular epithelium of plucked sheep hairs were successfully established. This was a simple technique where the plucked sheep hairs were attached directly to the bottom of plastic culture flasks and keratinocyte colonies formed without the aid of substrates or feeder layers. Initially, however, some difficulty was experienced in obtaining successful outgrowths from the plucked hairs. The outgrowth of keratinocytes is dependent upon the stage of growth that the hair follicle is in. The anagen phase of hair growth, where the hair has an almost intact epithelial outer root sheath acting as the source of the keratinocytes, is the most successful at producing keratinocytes colonies (Weterings *et al.*, 1981). In some studies the anagen phase hairs were selected microscopically and explanted for culture (Weterings *et al.*, 1981, Imcke *et al.*, 1987; Limat and Noser, 1986). In the present study however, all the plucked hairs, regardless of their stage of growth were explanted for culture as microscopic selection of anagen phase bulbs could not be readily accomplished aseptically. In order to increase the chance of obtaining primary cultures from follicles at unknown stages of growth, the number of hair follicles that were explanted into each culture flask was increased and the growth factors, hydrocortisone and epidermal growth factor (EGF) were included in the culture medium. Successful cultures of keratinocytes were subsequently obtained, the technique was reproducible and fibroblastic overgrowth was rarely observed. The concentrations of hydrocortisone and EGF added to the medium, were the concentrations typically used in human keratinocyte culture (Ogawa *et al.*, 1990, Grief *et al.*, 1988) and both have been reported to promote keratinocyte growth without fibroblastic overgrowth (Rheinwald and Green, 1975; Dover and Watt, 1987; Boyce and Ham, 1983; Pittlekow and Scott, 1986; Shipley and Pittlekow, 1987; Barnes and Sato, 1980). Typically EGF and hydrocortisone are included along with other growth factors such as cholera toxin (Dover and Watt, 1987) and insulin (O' Keefe and Chiu, 1988) as necessary constituents in serum-free medium used to grow keratinocytes. As EGF and hydrocortisone were not titrated in this study, their exact effect on ovine keratinocyte

growth was not determined. Human recombinant EGF was used which may not cross react with ovine EGF receptors. However it is possible that the addition of these growth factors to the culture medium reduced the risk of fibroblastic overgrowth. The direct explant of plucked hairs onto plastic cultures was reported not to be selective for keratinocytes (Wells *et al.*, 1985). The presence of serum within the medium will also promote keratinocyte growth as well as fibroblast growth. Both of these factors could have resulted in fibroblast contamination and yet fibroblastic overgrowth within the cultures was uncommon. The absence of fibroblasts from the primary cultures could be due to the lack of dermal components on the plucked hair and the promotion of keratinocyte growth by EGF and hydrocortisone.

Once established, the ovine keratinocyte cultures grew well and could be subcultured in the absence of EGF and hydrocortisone. Whilst the purity of the cultures could be judged by the slow growth rate and distinct morphology of the keratinocytes compared with fibroblasts, staining with anti-keratin and anti-vimentin confirmed these observations. The keratinocytes unlike the fibroblasts synthesised keratins that were detected as tonofilaments within the cytoplasm of approximately 99% of the cells in the cultures. Vimentin did not recognise keratinocytes and was observed to stain fibroblasts that formed the majority of the cells in the FL skin cultures. This technique of explanting plucked sheep hair follicles directly onto the bottom of plastic culture flasks without a feeder layer or substrate in the presence of EGF and hydrocortisone therefore appeared to be selective for keratinocytes, the only disadvantage being the limited numbers of cells that were obtained. As the cultured ovine keratinocytes were obtained from plucked hair follicles from the muzzle region of the sheep, the area typically infected by orf virus, they represent a good source of cells for *in vitro* studies of orf virus infection.

The initial *in vitro* studies of orf virus used a tissue culture adapted strain of virus, orf 11. This strain infects cells *in vitro,* but will not produce lesions *in vivo* (personal communication Dr. H.W. Reid). A second strain, Scabby Mouth was introduced into the *in vivo* work (see chapter 3) which does produce a lesion *in vivo*. As there is little known about differences in virulence between strains of orf virus, it was decided to

include Scabby Mouth in the *in vitro* work to determine any major differences between the two strains of virus. It became apparent that there were major differences in the induction of CPE by the two strains of orf virus. Scabby Mouth had an accelerated CPE that gave quite dramatic cell death by 12h post-infection. Orf 11 however, induced very limited CPE at 12h, the infected cultures differing from the control cultures by a slightly higher number of pyknotic cells. Orf 11 showed a disruption to the monolayer equivalent to that of 12h Scabby Mouth by 48h post-infection. Whilst there was a significant difference in the rate of appearance of CPE, there was no difference in the appearance of viral antigen for either strain. Viral antigen was detected in single cells at 12h post-infection, which for orf 11, occurred before the onset of CPE. The number of positive cells in the monolayer increased with time and by 48h virtually all the cells still attached to the culture vessel were positive for orf virus antigen. Both methods of viral antigen detection, immunofluorescence and immunoperoxidase, located the infected cells equally well, although immunofluorescence was more effective at highlighting the single infected cells at 12h post-infection. The monoclonal used in the immunoperoxidase method (5E2), which detected viral antigen in the cell *in vivo* (chapter 3) recognises an uncharacterised orf virus antigen. The hyperimmune serum used for immunofluorescent studies recognises 13 orf virus structural proteins (McKeever, 1987).

The production of infectivity by Scabby Mouth and orf 11 was not observed until 12h post-infection, which coincided with the detection of viral antigen. Both strains of orf produced infectivity that remained predominantly cell-associated until 72h post-infection when the viral 'burst' occurred, when most infectivity was released into the culture supernatants by lysis of the infected cells. Differences between the growth curves of Scabby Mouth and orf 11 were observed. Scabby Mouth replicated more rapidly than orf 11, with more infectivity being produced between 12h and 24h. Orf 11 however, appeared to be slower but more efficient at replicating in keratinocytes and produced greater infectivity than Scabby Mouth at 72h post-infection. It is possible that the slower CPE of orf 11 leaves a larger number of cell still viable and

capable of supporting viral replication, whilst Scabby Mouth infection results in rapid cell death leaving very few cells still attached to the flask at 48h post-infection. Despite the accelerated viral replication of Scabby Mouth, the CPE was observed before virus release and there was no difference in the expression of viral antigen between the two strains of orf virus. This indicates that there may be a toxic component in the Scabby Mouth inoculum that is affecting the cells, however the greater rate of replication of Scabby Mouth would suggest that this may not be the case emphasising the need to repeat this study using different batches of Scabby Mouth to confirm whether there is a significant difference in the cytopathogenicity of the two strains of orf virus; Scabby Mouth and orf 11.

The study of $^{35}S$ labelled amino acid incorporation by resting and orf virus infected keratinocytes was carried out to determine whether orf virus shuts down host cell protein synthesis. Vaccinia virus has the ability to switch off host cell protein synthesis within 30 minutes of infecting HeLa cells (Moss *et al.*, 1968). The present study measured protein that was synthesised and secreted into the culture supernatants within the first 12h post-infection with orf 11, where viral antigen, but not CPE was detected. Initial studies of cell lysates revealed a large number of protein bands making analysis difficult and so only the labelled proteins secreted into the supernatants were measured. Within the first 12h post-infection with orf virus, keratinocyte protein synthesis was not shutdown and instead appeared to be increased. Inhibition of viral protein synthesis was attempted by including actinomycin D (Shatkin 1963) in order to measure host cell protein synthesis alone. However actinomycin D was found to be quite toxic to keratinocytes at the concentrations used to inhibit vaccinia virus protein synthesis (Shatkin, 1963; Moss, 1968) and so it was included in the medium of two of the infected flasks at a low concentration of 1μg/ml. Whilst the ability of actinomycin D to inhibit viral protein synthesis or its affects on host cell protein synthesis was not determined, it did appear to reduce the level of protein synthesis by the infected cells. This reduction of protein synthesis could not be directly related to the inhibition of viral protein synthesis as it did not result in the disappearance of specific protein bands from the gel. The effect of actinomycin D on

orf virus and host cell protein synthesis needs to be determined and the experiment repeated using optimised conditions to identify viral protein bands. It was however concluded from this study that during the first 12h post-infection, orf virus appears to increase rather than decrease host cell protein synthesis.

In conclusion, this study established that pure cultures of ovine keratinocytes obtained from the follicular epithelium of plucked sheep hairs support orf virus replication. It was also observed that the two strains of virus orf 11 and Scabby Mouth, used in the studies differed in their ability to replicate and induce CPE. The first 12h post-orf virus infection represents an interesting area for subsequent study as during this period of time, in orf 11 infection there is no disruption to the cell monolayer, but orf viral antigen can be observed, coinciding with the detection of infectious virus particles and an increase in host cell protein synthesis. From 12h onwards, the cells are damaged by viral activity and become factories for the release of viral progeny. The next chapter will use this information to study cytokine production by keratinocytes over a time course following infection with orf virus.

# CHAPTER 5

## ANALYSIS OF CYTOKINE PRODUCTION BY OVINE KERATINOCYTES INFECTED WITH ORF VIRUS

## 5.1. INTRODUCTION

The target cell for orf virus replication *in vivo* is believed to be the keratinocyte (Jenkinson *et al.*, 1990). Keratinocytes, which represent more than 95% of the epidermal cell population, are a rich source of cytokines including IL-1, IL-3, IL-6, IL-8, GM-CSF, TNF$\alpha$, and transforming growth factors (Kupper, 1990; Damm *et al.*, 1989; Luger and Schwarz, 1990). Most studies on keratinocytes have been carried out using human and murine transformed cell lines; such as human epidermoid carcinoma cell lines and spontaneously transformed neonatal murine keratinocyte-derived (Pam 212) cells. The constitutive production of cytokines by these cells is typically very low but can be significantly increased by various injurious agents such as endotoxin, tumour promoters (for example, phorbol myristate acetate, TPA), mitogens (for example, concanavalin A), ultraviolet irradiation (UV) or viral infection (Kupper *et al.*, 1990, Damm *et al.*, 1989 and Luger and Schwarz, 1990).

With regard to the immunological control of cutaneous viral infections, keratinocytes and LC have previously been shown to play a critical role in the mechanisms of defence against herpes simplex virus type-1 (HSV-1). Both cell types have been hypothesised to be able, not only to trigger a systemic immune response to HSV-1, by presenting viral antigens to T cells, but also to oppose virus progression by local secretion of cytokines with anti-viral activity (Sprecher and Becker, 1986, 1987, 1989). The cytokines, IL-1 and TNF$\alpha$, display strong anti-viral activity and messenger RNA (mRNA) for both has been shown to be induced in murine keratinocytes infected with HSV-1 (Sprecher and Becker, 1992). Orf virus, like HSV-1, is characterised by its epidermotropism. The ability of orf virus to re-infect the host, despite the presence of specific anti-viral antibodies, has recently focused attention on the importance of the local, cellular immune response at the site of the infection. It might be envisaged, therefore, that ovine keratinocytes infected with orf virus produce a number of cytokines *in vivo* that modulate Langerhans cell function, induce inflammation and prevent the replication and spread of the virus in the host. Alternatively, or in addition, it may be posible that the infection induces an alteration

in the normal cytokine profile of keratinocytes leading to the prolongation of the orf lesion *in vivo*, ineffective clearance of the virus from the local site, and the chance of developing lesions again on re-infection. Thus the overall aim of this study was to characterise the cytokines produced by keratinocytes infected by orf virus *in vitro* which may play an important role in the local immune response to orf virus *in vivo*.

Cytokine production by cultured ovine keratinocytes has not been previously analysed. Thus, in order to characterise the cytokines produced by ovine keratinocytes and the sensitivity of the detection systems used to measure their production, fresh cultures of keratinocytes were analysed for their ability to produce cytokines under normal, resting conditions and after stimulation with TPA or UV irradiation. Keratinocytes were then infected *in vitro* with orf virus and cytokine production measured at specific time points post-infection. Cytokine expression was assayed in terms of both mRNA and protein, wherever possible.

## 5.2. RESULTS

In this study, cultures of ovine keratinocytes were first analysed for their ability to produce a number of cytokines under resting culture conditions and in response to two stimuli known to induce cytokine production by human and murine keratinocytes, namely incubation with TPA/calcium ionophore, and UV irradiation. The main part of the study was then to determine the effect of orf virus infection on the profile of cytokines produced by ovine keratinocytes in culture. Cytokine production by keratinocytes was measured at two levels; at the level of transcription, using an RT-PCR technique to detect cytokine mRNA, and at the level of protein, using ELISAs and bioassays developed for the detection of ovine cytokines.

### 5.2.1. TPA/calcium ionophore stimulated keratinocytes

Confluent monolayers of keratinocytes were incubated with 5 ng/ml of TPA and 500ng/ml of calcium ionophore in keratinocyte culture medium containing 6% FCS. The supernatants and cellular RNA were collected from control and stimulated cells at 0h, 4h, 8h and 12h after stimulation, and were assayed for various cytokines as outlined below.

*i). IL-8*

Production of IL-8 by keratinocytes was examined by measuring its concentration in the culture supernatants by ELISA. Supernatant samples from the 0h control (unstimulated) and 0h TPA stimulated cultures contained low levels of IL-8 which could also be detected in the keratinocyte culture medium itself. IL-8 was found to be constitutively produced by the control, unstimulated keratinocytes as the concentration of IL-8 in the culture supernatants increased steadily over the 12 hour incubation period (Figure 5.1). The stimulation of keratinocytes by TPA/calcium ionophore resulted in a marked increase in the production of IL-8 by the cells (Figure 5.1). At each time point analysed post-stimulation, the stimulated cells had markedly higher levels of IL-8 in their supernatants when compared with the unstimulated controls.

Neat supernatants gave high readings which were out of the ELISA range and so the results shown are for 1:3 dilutions of supernatants and are representative of two independent ELISA tests of the same samples carried out in duplicate.

As the primers and probes were not available for IL-8, the induction of mRNA by TPA/calcium ionophore could not be correlated with the induction of IL-8 protein measured above.

### *ii). GM-CSF*

The concentration of GM-CSF in the supernatants of resting and stimulated keratinocytes was measured by ELISA. The keratinocyte culture medium itself contained a negligible amount of GM-CSF. Constitutive production of GM-CSF was observed in the unstimulated cells with a steady accumulation of GM-CSF being detected in the culture supernatants over the 12h collection period (Figure 5.2). TPA stimulation did not appear to increase the production of GM-CSF by keratinocytes as the concentration of GM-CSF in the supernatants was no higher than in the unstimulated cells (Figure 5.2). The production of GM-CSF mRNA gave a good correlation with GM-CSF released into the supernatants. Initially at 0h, GM-CSF mRNA was not detected (Figure 5.3) but by 4h mRNA was present in both the control and stimulated cells and remained so in both cultures up to the last time-point examined (12h).

### *iii). Cytokines TNFα, IFN, IL-1β and IL-3*

The supernatants from resting and TPA/calcium ionophore stimulated keratinocytes were tested for TNFα and IFN in bioassays and for IL-1β in an ELISA. The results for all three cytokines for both resting and TPA stimulated keratinocytes were negative. Very faint bands on the blots for TNFα mRNA were observed for both stimulated and unstimulated cells (Figure 5.3) suggesting that the amount of TNFα

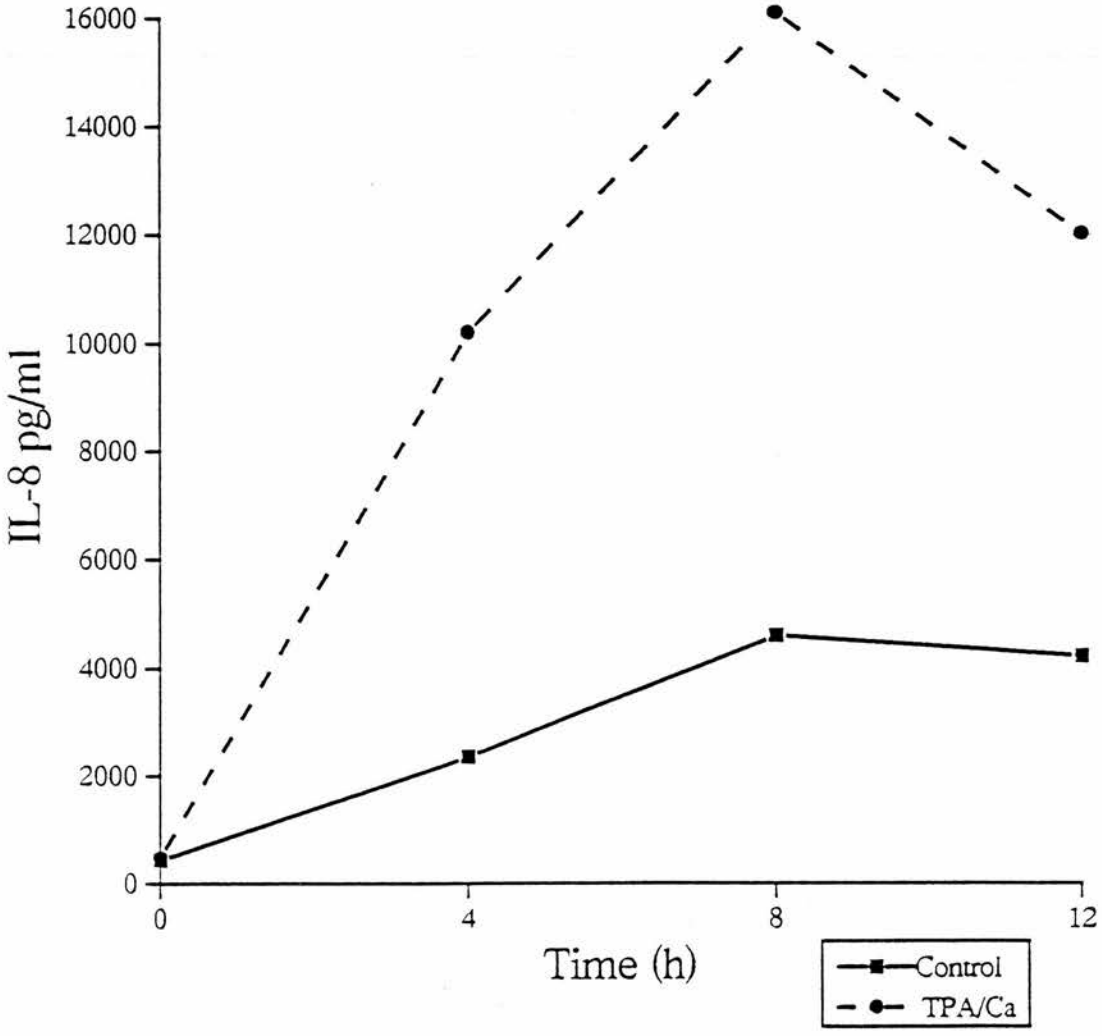



**Figure 5.1** *The effect of TPA/calcium ionophore treatment on keratinocyte production of IL-8. The supernatants from the control and treated (TPA/Ca) cultures were assayed for IL-8 protein by ELISA at 4h intervals until 12h post-treatment.*

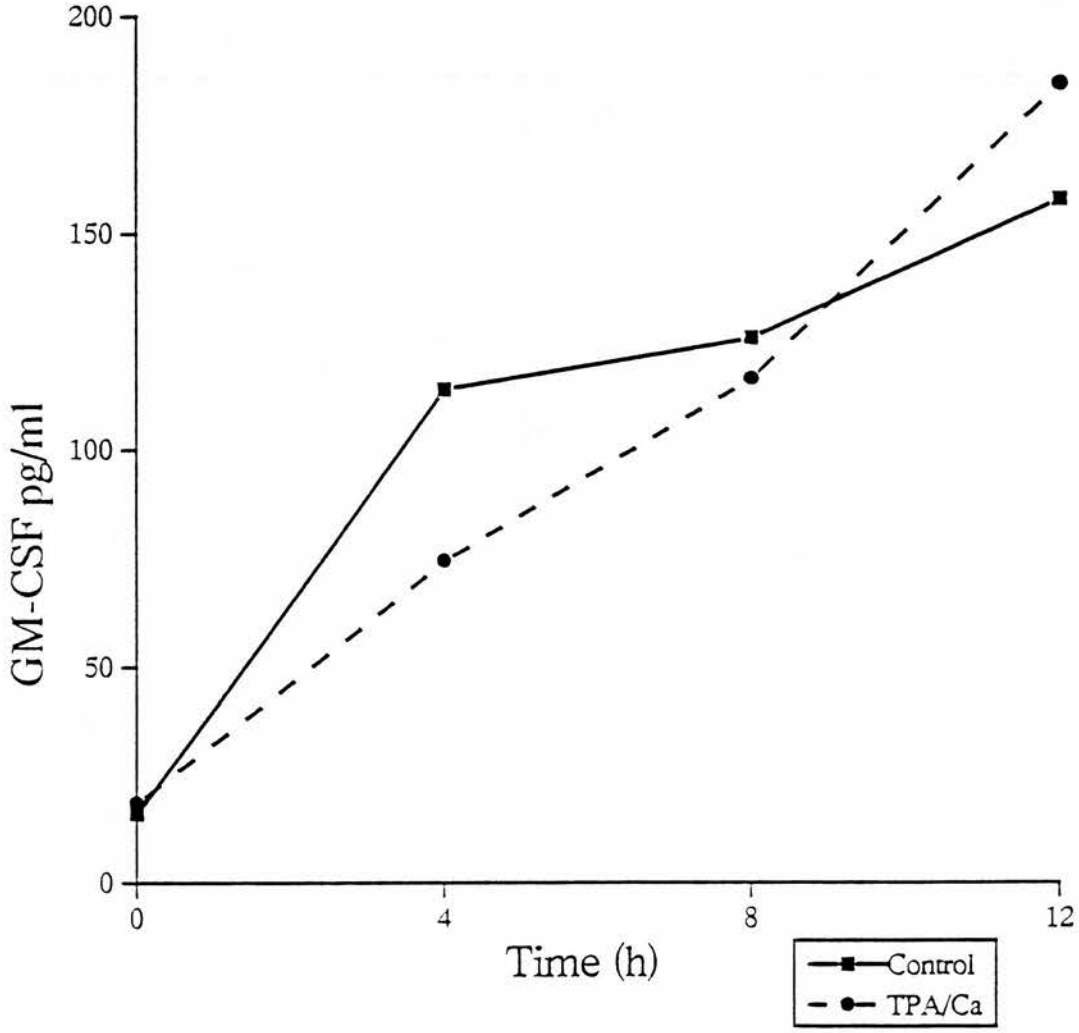



**Figure 5.2** *The effect of TPA/calcium ionophore treatment on keratinocyte production of GM-CSF. The supernatants from the control and treated (TPA/Ca) cultures were assayed for GM-CSF protein by ELISA at 4h intervals until 12h post-treatment.*

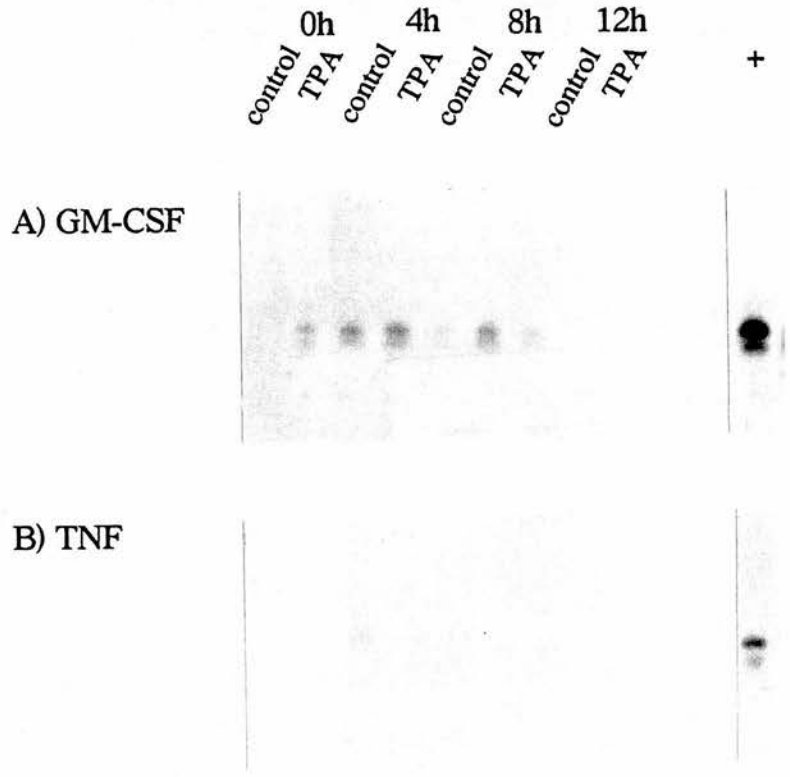



**Figure 5.3** *The effect of TPA/calcium ionophore treatment on* **A)** *GM-CSF and* **B)** *TNFα. mRNA production. mRNA for control and treated (TPA/Ca) cultures was analysed by RT-PCR at 4h intervals until 12h post-treatment. Positive controls were included for each cytokine.*

produced by the cells may be too low for the level of detection of the bioassay. mRNA was not detected for either IL-1β or IFN- γ or IL-3.

### 5.2.2. UV irradiated keratinocytes

Cytokine production by monolayers of keratinocytes irradiated with UV light was analysed. The keratinocytes were exposed to three types of UV light; UVB broad band (270nm-320nm) at doses of 20mJ/cm2 or 10mJ/cm$^2$, UVB narrow band (312nm) at doses of 50mJ/cm$^2$ or 20mJ/cm$^2$ and UVA (310-400nm) at a single dose of 100mJ/cm$^2$, which was then followed by incubation for 24 hours in keratinocyte culture medium.

*i). IL-8*

IL-8 production by UV-irradiated keratinocytes was not studied as the ELISA only became available after the experiment was completed.

*ii). GM-CSF*

The number of cells used in the experiment was lower than in the TPA/calcium ionophore experiment and so the levels of GM-CSF detected within the culture supernatants by ELISA can only be compared with the non-irradiated, control group. The results for the experiment are shown in Figures 5.4 and 5.5. Constitutive production of a small amount of GM-CSF was seen in the supernatants of the control cells (Fig 5.4) which correlated with the low production of mRNA (Figure 5.5) and was consistent with previous findings. UVB broad band irradiation resulted in a marked increase in the production of GM-CSF mRNA for both the high and low doses (Figure 5.5), with the strongest band detected in the high dose irradiated cells. The increased mRNA correlated with a dramatic increase in the level of GM-CSF detected in the culture supernatant of the cells receiving the low dose only, the supernatants from the keratinocytes that received the high dose contained only low levels of GM-CSF. The high dose of UVB light resulted in visible damage (cell rounding) to the cell monolayers that was not observed at the low dose and it is

possible that such a high dose inhibits translation of GM-CSF or that the cells are killed before translation can occur. Neither the UVB narrow band, high or low doses, or UVA had any effect on the production of GM-CSF, with the concentrations of GM-CSF protein not differing from the controls. This result was reflected in the low levels of mRNA observed for each treatment (Figure 5.5, lanes 3, 4 and 5).

### *iii). Cytokines TNFα, IFN, IL-1β and IL-3*

The bioassays for the detection of TNFα and IFNs and the ELISA for IL-1β gave negative results for all irradiated groups. Similarly, no mRNA for the above cytokines could be detected in any of the groups. A faint mRNA signal for the cytokine IL-3 could be detected in the cells irradiated with UVB broad band high and low doses (not shown) but no bioassay or ELISA was available to confirm these results.

## 5.2.3. Orf virus infected keratinocytes

The previous experiments showed that, under normal culture conditions, ovine keratinocytes constitutively produced the cytokines IL-8 and GM-CSF. The production of these cytokines by control keratinocytes was found to correlate with the concentration of foetal calf serum (FCS) in the culture medium. When the FCS was reduced from 6% to 1%, the concentration of IL-8 produced by the cells after 24h fell from approximately 18ng/ml to about 0.2ng/ml. Similarly the concentration of GM-CSF produced by the cells after 24h was reduced from 200 pg/ml (6% FCS) to a concentration that was below the limit of detection of the assay (1% FCS). In order to study the effect of orf virus infection on keratinocyte cytokine production, it would have been preferable to culture the keratinocytes in serumless medium. However several attempts to maintain cells in different serumless formulations of keratinocyte culture medium from commercial companies were unsuccessful. Thus keratinocytes were infected with orf virus (Scabby Mouth or orf 11) in culture medium containing 1% FCS and cytokine production analysed at various times, up till 24h post-infection.

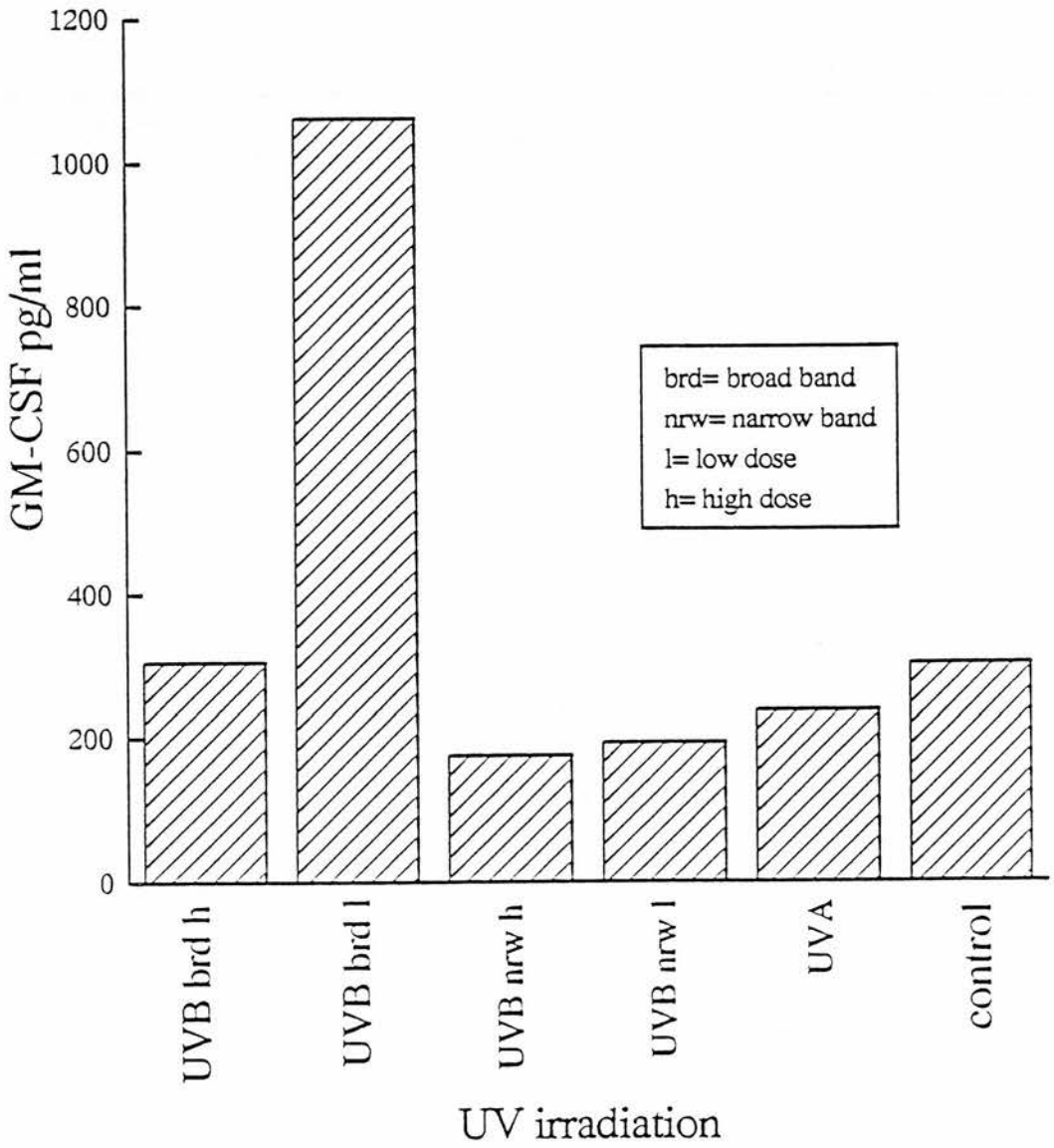



**Figure 5.4** *The effect of UV irradiation on keratinocyte production of GM-CSF. The supernatants from the control and UV irradiated cultures were assayed for GM-CSF protein by ELISA 24h post-treatment.*

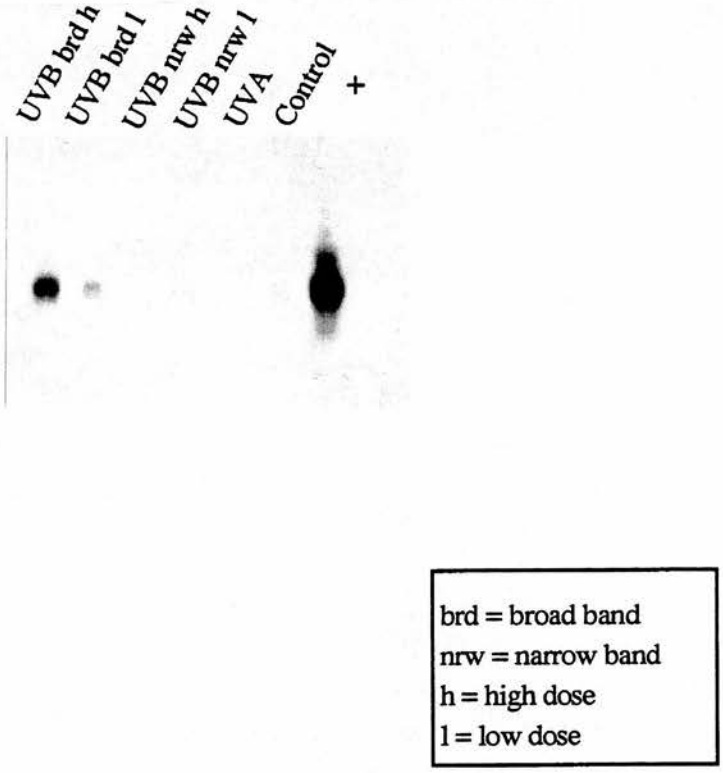



**Figure 5.5** *The effect of UV irradiation on the production of GM-CSF mRNA. mRNA was analysed by RT-PCR and a GM-CSF mRNA positive control included.*

*i). IL-8*

Constitutive production of IL-8 was observed, with low concentrations accumulating within the control culture supernatants from 2h onwards (Figure 5.6). In response to infection with orf virus (both Scabby Mouth and orf 11), IL-8 production was stimulated, and increased amounts of IL-8 accumulated within the supernatants compared with the controls (Figure 5.6). Initially there was no difference between the response of the cells to the two strains of orf viruses. Increased production of IL-8 occurred from 2h post-infection and by 10-12h post-infection with orf virus, a seven fold increase in the concentration of IL-8 was observed in the infected supernatants compared with the equivalent controls. However, between 12 and 24h post-infection, the level of IL-8 in the supernatant from the keratinocytes infected with Scabby Mouth decreased dramatically and was probably associated with the accelerated CPE observed for Scabby Mouth. The CPE for orf 11 was not as advanced at 24h and the supernatants from these cells contained the highest concentration of IL-8 detected for the infected samples.

*ii). GM-CSF*

The reduction of the serum in the culture medium to 1% abolished the constitutive production of GM-CSF by the control, uninfected keratinocytes. No GM-CSF could be detected in the culture supernatants and only very low GM-CSF mRNA was detectable (Figure 5.7). Similarly, there was no detectable GM-CSF in the orf infected culture supernatants at any of the timepoints studied. Despite this, however, mRNA analysis showed increased production of GM-CSF mRNA in orf virus (orf 11 and Scabby Mouth) infected keratinocytes at 2h and 4h post-infection (Figure 5.7).

In order to establish whether the absence of GM-CSF from the supernatants of the infected cells was purely due to the lack of stimulus from the FCS, cultures of keratinocytes were infected with orf virus and incubated in medium containing 2% FCS. As there was no detectable difference in the response to either of the strains of orf virus, the experiment was carried out using orf 11 only.

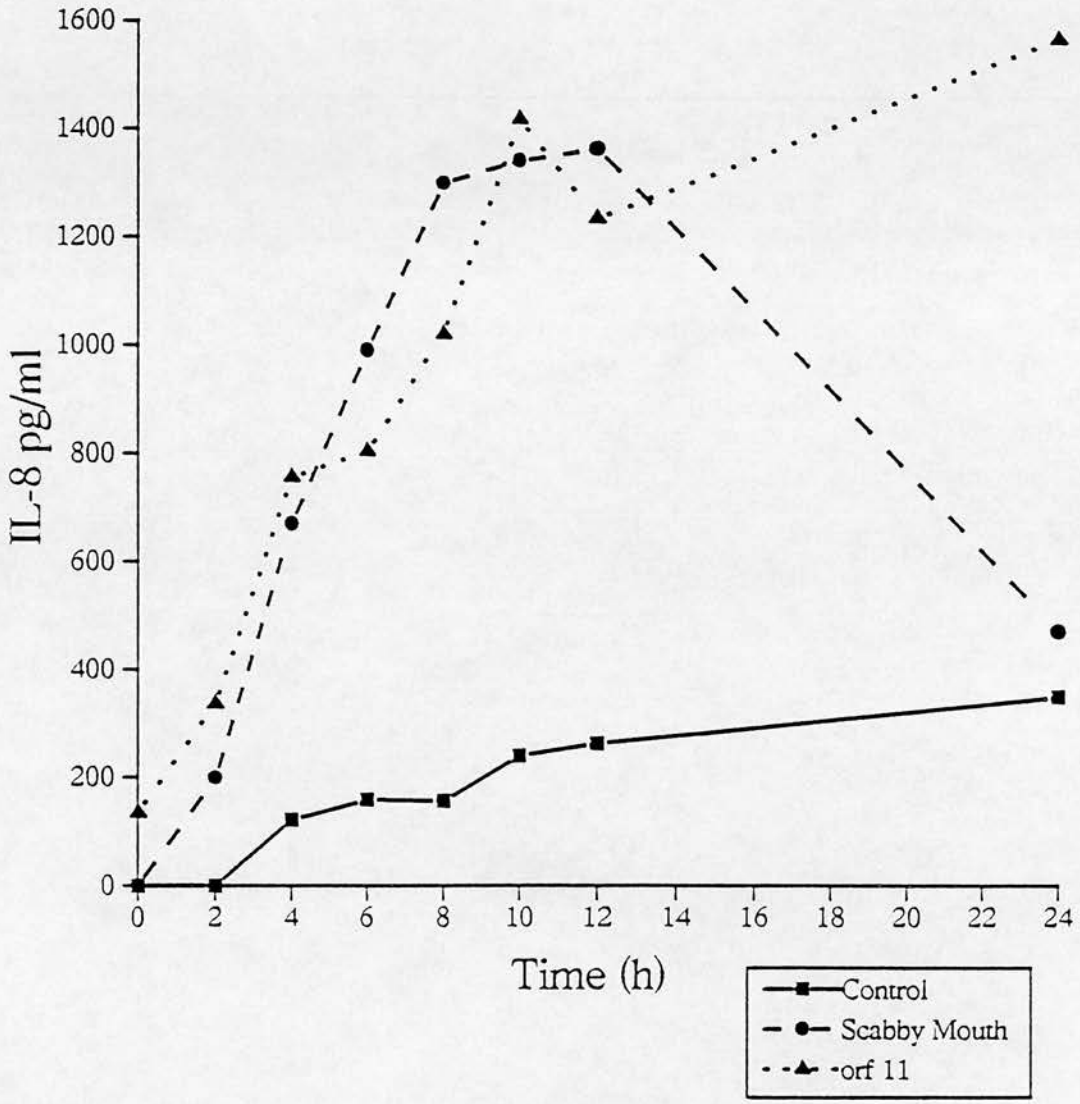



**Figure 5.6** *The effect of orf virus infection on keratinocyte production of IL-8 in the presence of 1% FCS. The supernatants from control cultures and cultures infected with Scabby mouth or orf 11 virus were assayed for IL-8 protein by ELISA at 2h intervals until 12h post-treatment, and subsequently at 24h post-infection.*

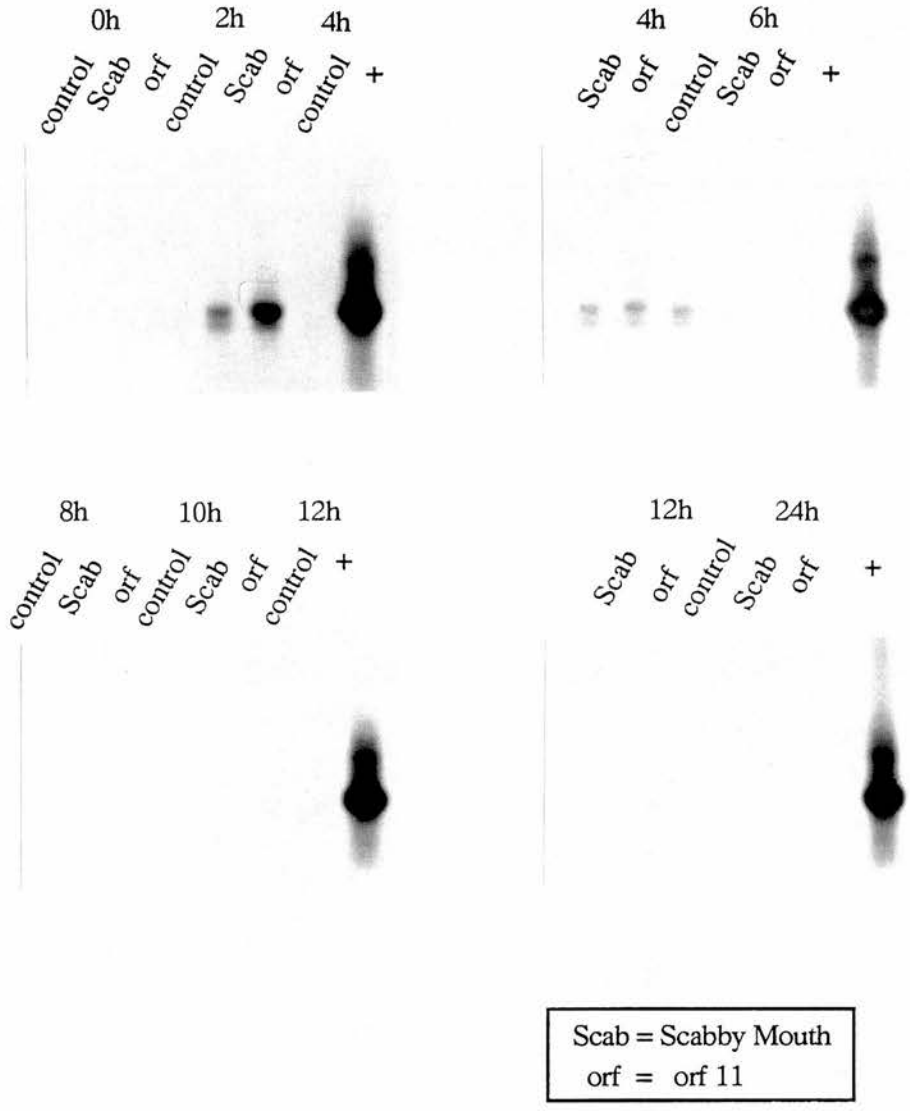



**Figure 5.7** *The effect of orf virus infection on GM-CSF mRNA production in the presence of 1% FCS. GM-CSF mRNA was analysed by RT-PCR for control cultures and cultures infected with Scabby Mouth or Orf 11 virus at 2h intervals until 12h post-infection, and subsequently at 24h post-infection.*

The increase in the concentration of FCS stimulated the production of GM-CSF by the control, uninfected cells (Figure 5.8), as was expected. The accumulation of GM-CSF within the control, culture supernatants was reflected in the production of high levels of GM-CSF mRNA at each timepoint (Figure 5.9). However, although some GM-CSF was produced by the infected cells, the concentration was much lower than the amount produced in the equivalent control cultures (Figure 5.8). Analysis of mRNA from the infected cells showed little difference to the results observed for the controls (Figure 5.9). Thus in the first 12h post-infection, the level of transcription of GM-CSF by the infected cells was equivalent to that of the controls but did not seem to correlate with the low amount of GM-CSF protein present within the infected culture supernatants. It appeared, therefore, that during the first 12h post-infection, orf virus inhibited the production of GM-CSF by keratinocytes at a level beyond the transcription of mRNA. A decrease in the level of GM-CSF mRNA production was only detected at 24h post-infection.

### *iii). β-actin mRNA*

β-actin mRNA production by control and orf virus infected cells was analysed and included as a positive control for gene transcription by the cells. The results showed that during the first 12h post-orf virus infection, there was no effect on the ability of the cells to transcribe the β-actin gene into mRNA (Figure 5.10). Even at 24h post-infection when GM-CSF mRNA was decreased the β-actin mRNA did not appear to be affected by Scabby Mouth or orf 11 infection.

### *iv). Cytokines TNFα, IFN, IL-1β and IL-3*

The bioassays for the detection of TNFα and IFNs were both negative for all samples analysed. The samples were also tested for TNFα in a more sensitive radioimmunoassay. A very low concentration of TNFα was detected in the supernatants at 0h from both control and infected cultures. A small increase in TNFα

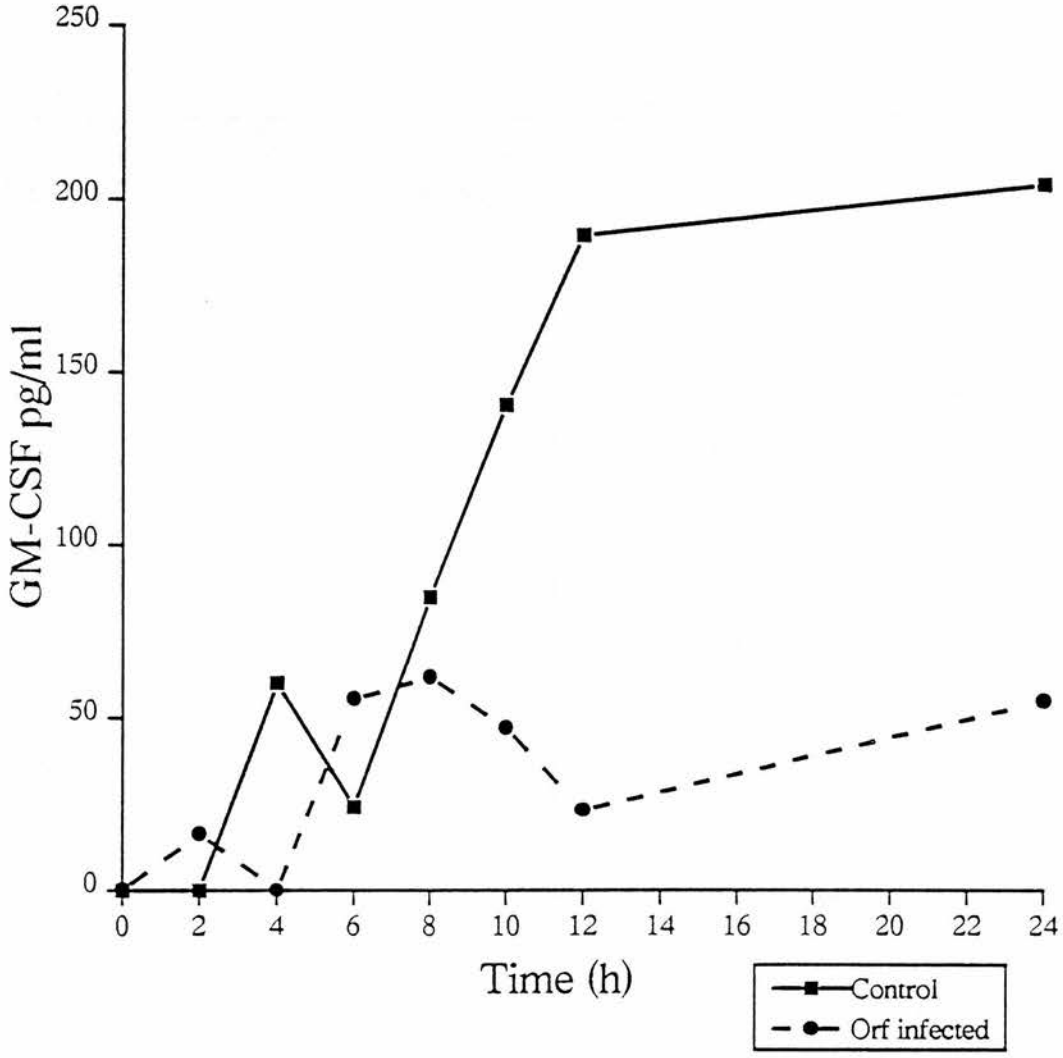



**Figure 5.8** *The effect of orf virus infection on keratinocyte production of GM-CSF, in the presence of 2% FCS. Orf 11 virus infected keratinocytes were assayed for GM-CSF protein by ELISA at 2 hour intervals until 12h post-infection, and subsequently at 24h post-infection.*

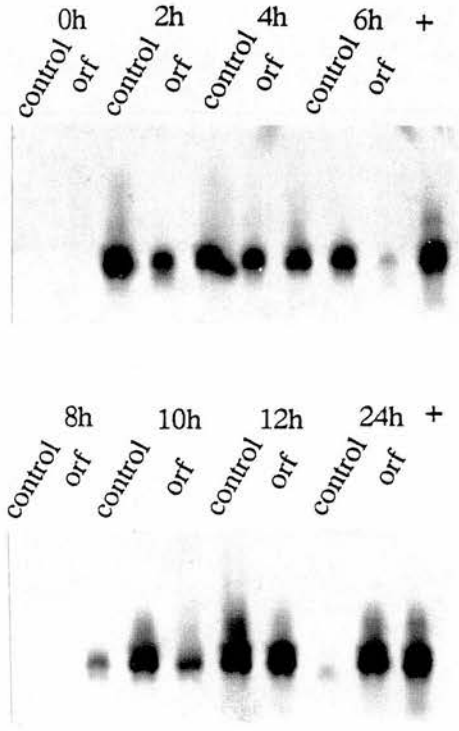



**Figure 5.9** *The effect of orf virus infection on GM-CSF mRNA production in the presence of 2% FCS. GM-CSF mRNA was analysed by RT-PCR for control cultures and cultures infected with orf 11 virus at 2h intervals until 12h post-infection and subsequently at 24h post-infection.*

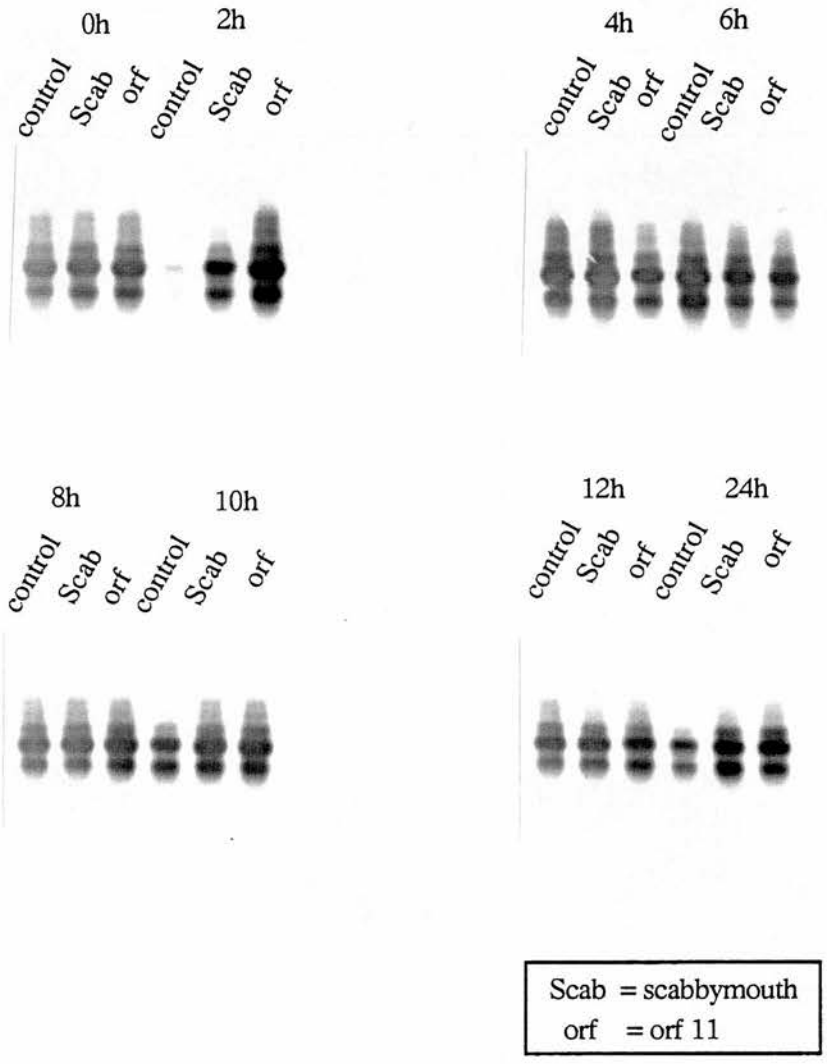



**Figure 5.10** *The effect of orf virus infection on β-Actin mRNA production in the presence of 1% FCS. β-actin mRNA was analysed by RT-PCR for control cultures and cultures infected with Scabby Mouth or orf 11 virus at 2h intervals until 12h post-infection and subsequently at 24h post-infection.*

was detected between 6 and 12 hours in the infected supernatants but this was also observed in the uninfected control supernatants. TNFα in both the control and infected supernatants then declined between 12 and 24 hours to levels at the limit of detection. mRNA for TNFα could be detected in some of the samples (infected and uninfected), but, the results were very variable with no obvious pattern making it difficult to come to any conclusions (see Figure 5.11). IL-1β could not be reproducibly detected within the supernatants by ELISA which correlated with the absence of IL-1β mRNA for control and infected cells at all timepoints studied. The presence of mRNA for IL-3 was occasionally detected in the infected cell cultures. However the results were not reproducible, and as there was no assay available to measure the concentration of IL-3 protein in the culture supernatants, no conclusions could be reached about the production of IL-3 by the infected keratinocytes

*v). Clearance of GM-CSF from supernatants of orf infected keratinocytes*

As it appeared from the data in Figure 5.8 that orf virus infection of keratinocytes inhibited the production of GM-CSF, further experiments were carried out to ascertain whether there were any inhibitory factors produced by the infected cells that interfered with the detection of GM-CSF in culture supernatants.

Exogenous, recombinant ovine GM-CSF at a concentration of 4000pg/ml was added to cell-free culture supernatants collected from control and infected cells and then, after incubation, the samples were diluted (as for the ELISA standards) and the ELISA carried out as before. The OD at 492nm for each of the control and infected supernatants was read and plotted against the amount of GM-CSF that was contained within each dilution. The results are shown in Figure 5.12A, B and C, and the standard curve for GM-CSF prepared in diluting buffer, included in each. GM-CSF incubated with supernatants collected from orf virus infected keratinocytes was found to be reduced in concentration when measured in the ELISA. This effect was specific for the infected supernatants only and this reduction in GM-CSF increased with the progression of infection.

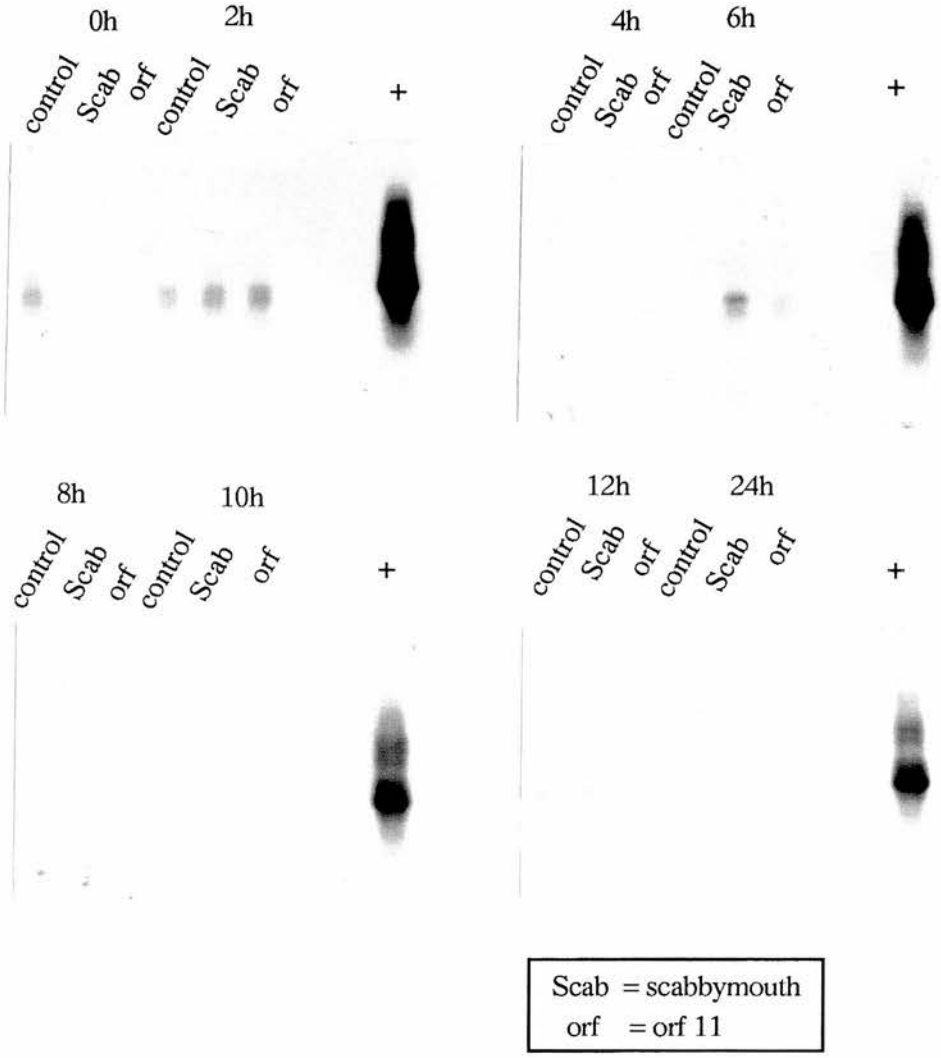



**Figure 5.11** *The effect of orf virus infection on TNFα mRNA production in the presence of 1% FCS. TNFα mRNA was analysed by RT-PCR for control cultures and cultures infected with Scabby Mouth or orf 11 virus at 2h intervals until 12h post-infection and subsequently at 24h post-infection.*

Figure 5.12A shows that there was no obvious difference in the concentration of GM-CSF, as measured by the ELISA, between the 0h control and 0h infected supernatants and the standard curve. Using supernatants collected from 2h post-infection, the concentration of the exogenous GM-CSF became gradually lower, with the curves for each of the infected supernatants falling away from the standard curve. Figure 5.12B shows that from 4h-10h post-infection the inhibition of GM-CSF measurement does not increase dramatically but by 12h and 24h post-infection there is a marked reduction from the levels of GM-CSF expected from the standard curve (Figure 5.12C). The GM-CSF in the 12h and 24h control supernatants was unaffected and produced results similar to the standard curve (Figure 5.12C). The results described above were for supernatants from orf 11 infected cells in the presence of 2% FCS. Supernatants from keratinocytes infected with both Scabby Mouth and orf 11 cultured in the presence of low and high serum were found to exhibit the same effect when tested in the inhibition ELISA, with the highest inhibition detected at 24h post-infection (data not shown).

### *vi). The effect of heating and protease inhibitors on the clearance of GM-CSF from infected culture supernatants*

Two further experiments were carried out to identify the possible nature of the factors involved in the clearance of GM-CSF from orf infected supernatants. Control and orf infected supernatants were heated or mixed with protease inhibitors before the addition of a known quantity of exogenous GM-CSF (as described previously). The ELISA was then carried out to determine whether the process of GM-CSF clearance could be abolished by either of these steps.

#### *Heating*

Cell-free supernatants from 12h and 24h control and orf virus infected cultures were heated to 56°C for 1 hour before the addition of exogenous GM-CSF. The concentration of GM-CSF (pg/ml) detected from the 4000pg/ml added, was plotted

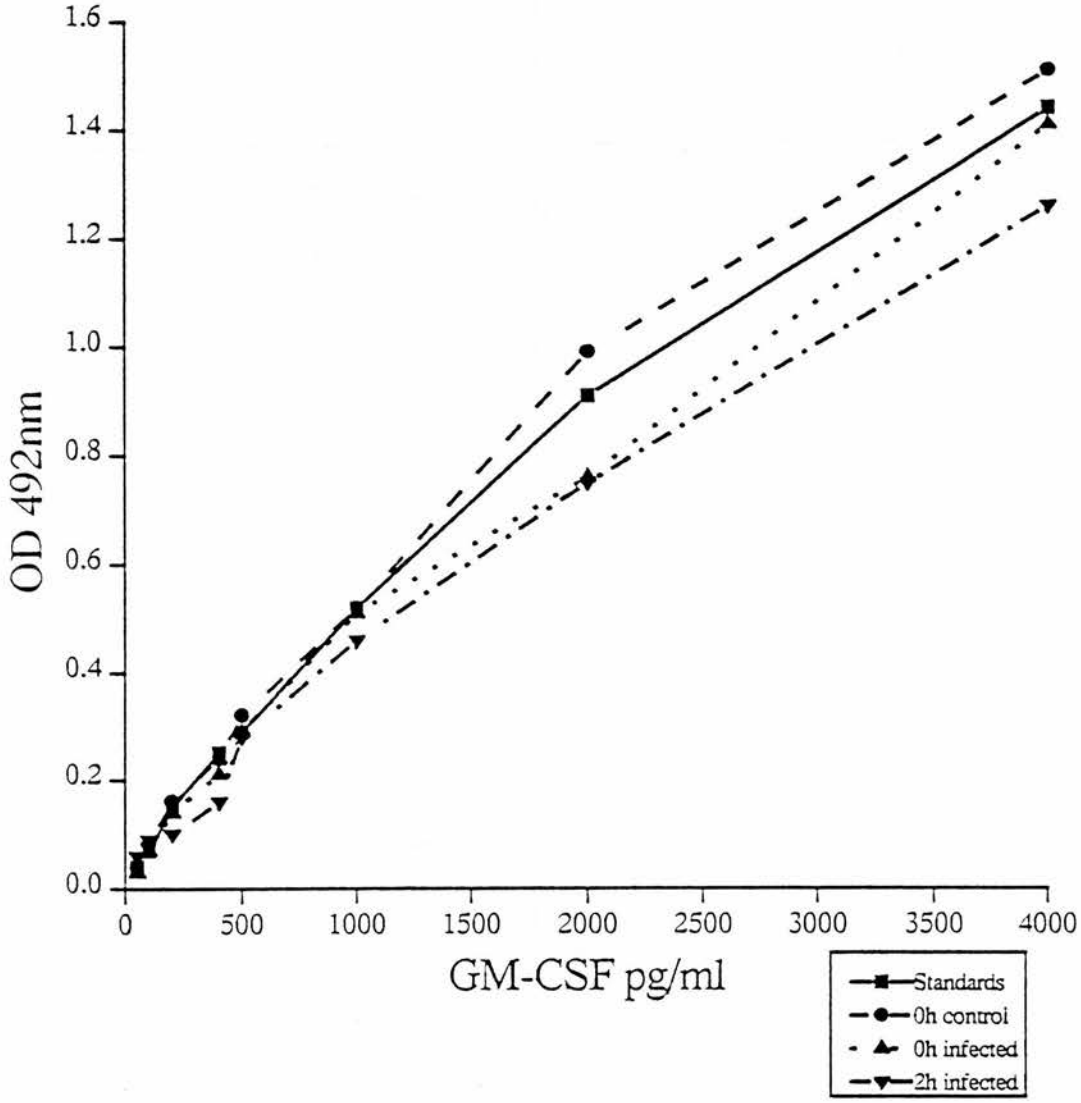



**Figure 5.12A** *The standard curves produced after the incubation of 4000pg/ml of exogenous recombinant ovine GM-CSF in the supernatants collected from oh control keratinocytes and keratinocytes infected with orf 11 virus (at 0h, and 2h post-infection). Standards prepared in buffer were also included.*

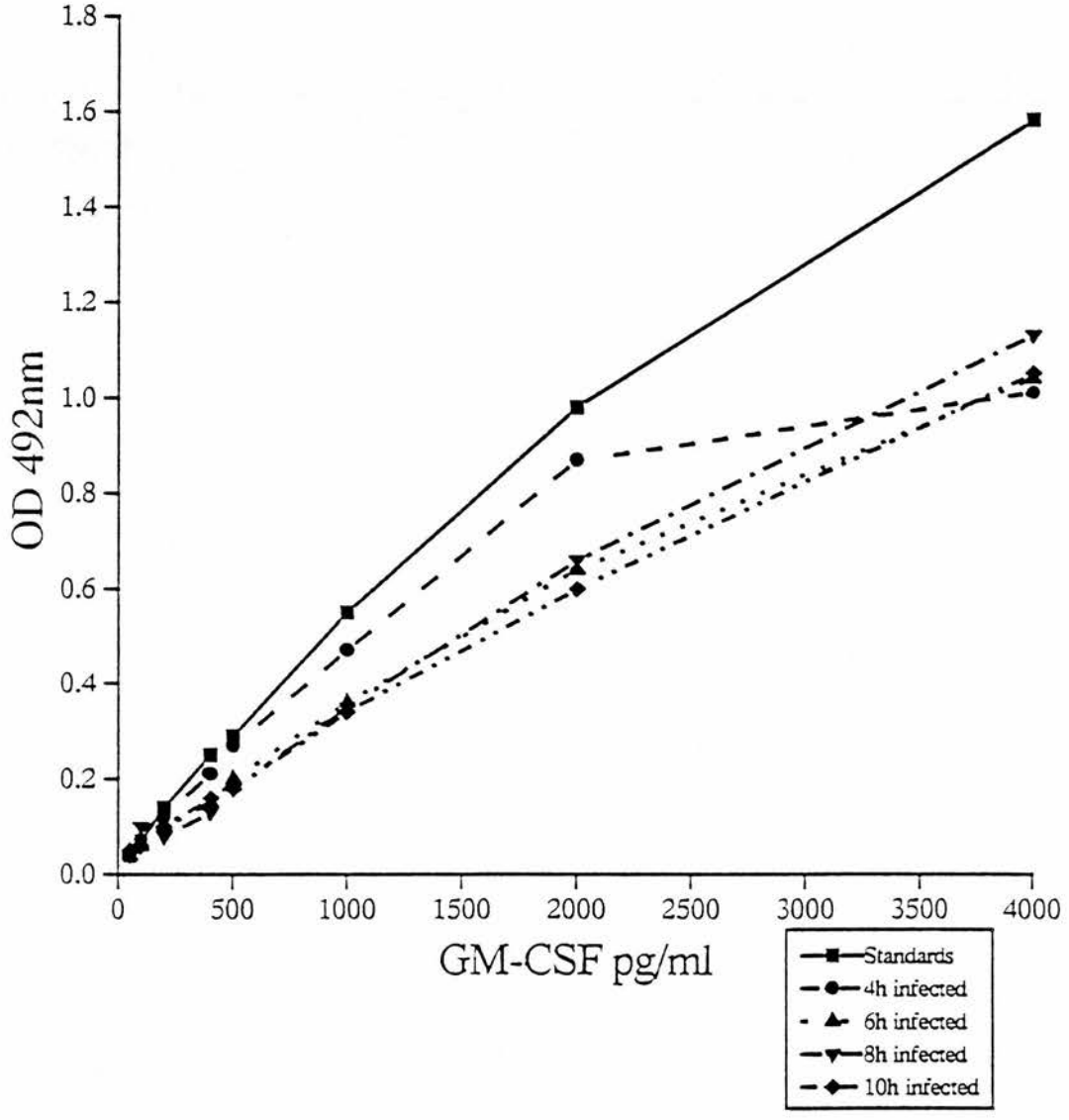



**Figure 5.12B** *The standard curves produced after the incubation of 4000pg/ml of exogenous recombinant ovine GM-CSF in the supernatants collected from keratinocytes at 4h, 6h and 8h post-infection with orf 11 virus. Standards prepared in buffer were also included.*

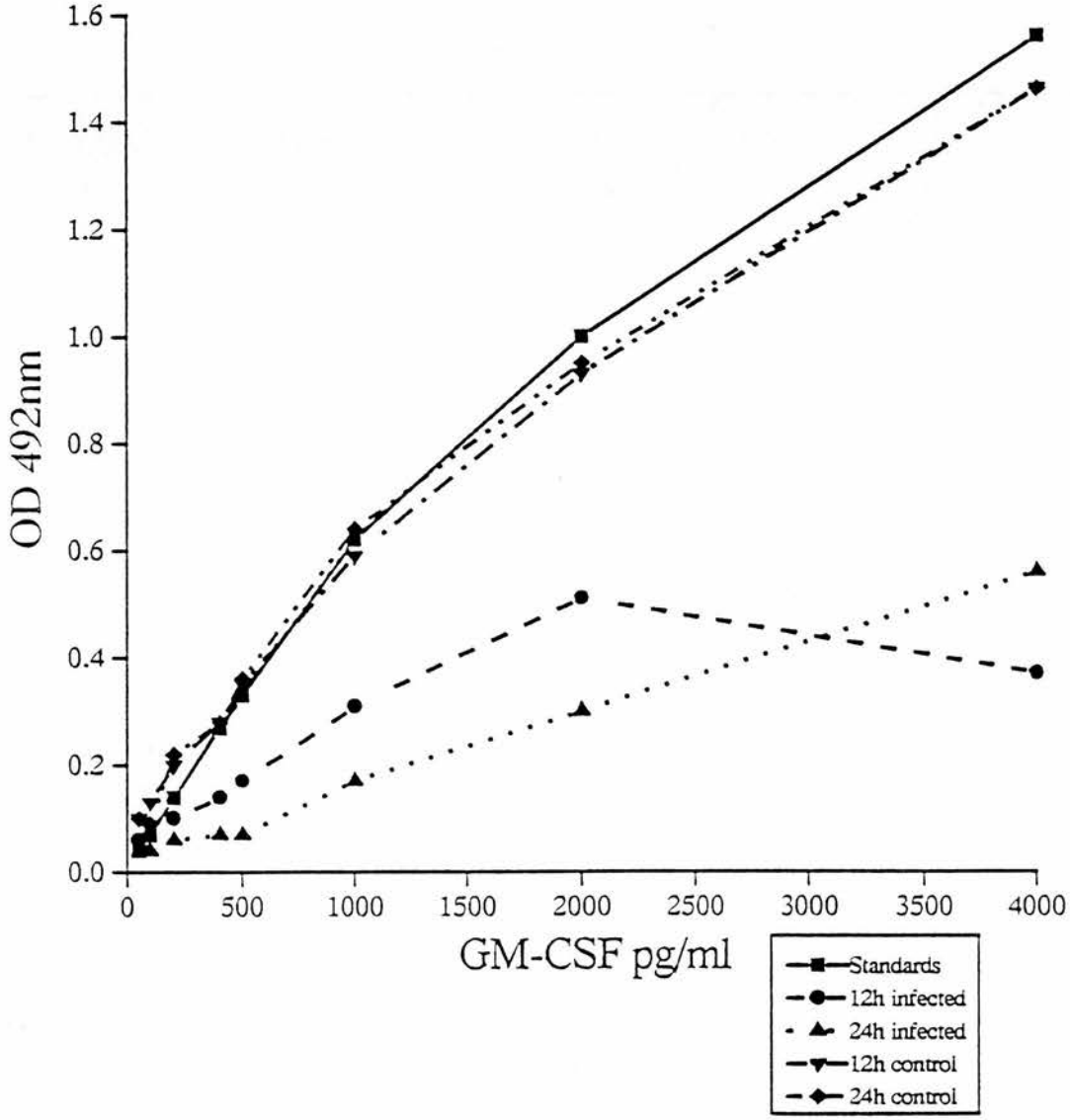



**Figure 5.12C** *The standard curves produced after the incubation of 4000pg/ml of exogenous recombinant ovine GM-CSF in the supernatants collected from12h and 24h control and orf 11 virus infected keratinocytes. Standards prepared in buffer were also included.*

as a bar chart and the results shown in Figure 5.13. The 12h and 24h control supernatants did not inhibit the GM-CSF, the readings for both untreated and heated supernatants varying slightly around the 3000-4000 pg/ml level (Figure 5.13). The process of heating was found to affect the ability of the infected supernatants to clear GM-CSF. The 12h unheated orf infected supernatant contained approximately 1600 pg/ml detectable by the ELISA, whereas after heating approximately 4000pg/ml could be detected corresponding to the amount of GM-CSF added (Figure 5.13). Heating the 24h infected supernatant only partially reversed the inhibitory effect - the unheated sample gave a reading of less than 1000 pg/ml of GM-CSF whereas, after heating, 1500 pg/ml was detectable (Figure 5.13).

*The effect of protease inhibitors on the clearance of GM-CSF*

Protease inhibitors that stop the action of the four main types of proteases; aspartate proteases, cysteine proteases, metalloproteases and serine proteases (see table 2.3, chapter 2) were incubated individually with the control and 24h orf virus infected supernatants before the addition of exogenous GM-CSF to determine whether the clearance of GM-CSF could be attributed to protease activity. The ELISA was carried out as before and untreated control and infected samples were included. The inhibition of protease activity within the infected supernatant for all four types of proteases, had no effect on the ability to interfere with the detection of GM-CSF as both treated and untreated 24h infected supernatants reduced the amount of GM-CSF to the same extent (Figure 5.14).

## 5.2.4. Analysis of the proteolytic activity of keratinocytes

The supernatants and cell lysates collected for the $^{35}S$ incorporation experiment described in chapter 4 were also examined for their proteolytic activity. The supernatants were concentrated ten-fold and both supernatants and cell lysate preparations analysed on aso-casein gels.

All of the cell lysate preparations collected from both control and orf virus infected keratinocytes contained proteolytic activity detected by a band of degradation of the

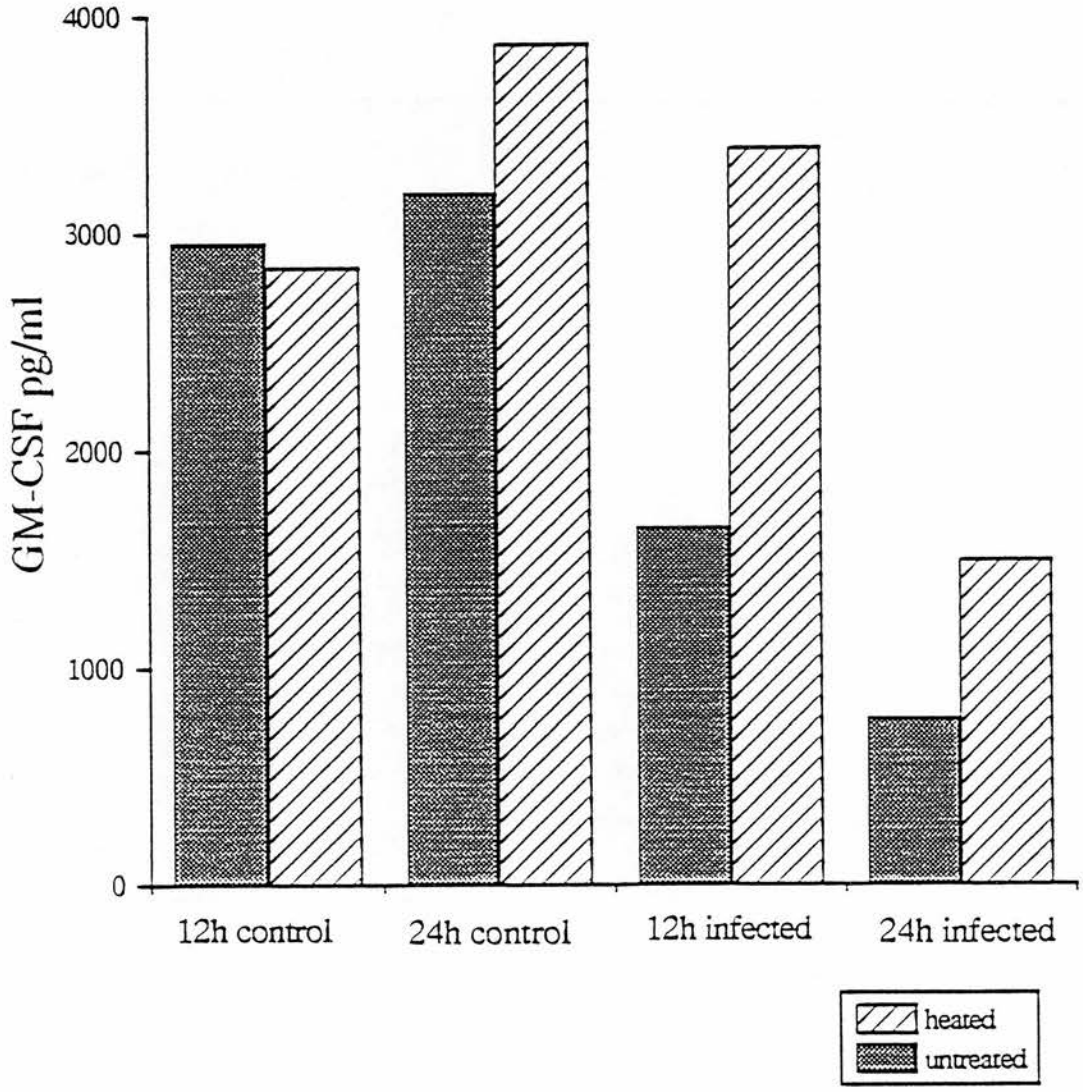



**Figure 5.13** *The effect of heating on the clearance of recombinant ovine GM-CSF by culture supernatants collected from keratinocyte cultures at 12h and 24h post-infection with orf 11 virus and equivalent control cultures.*

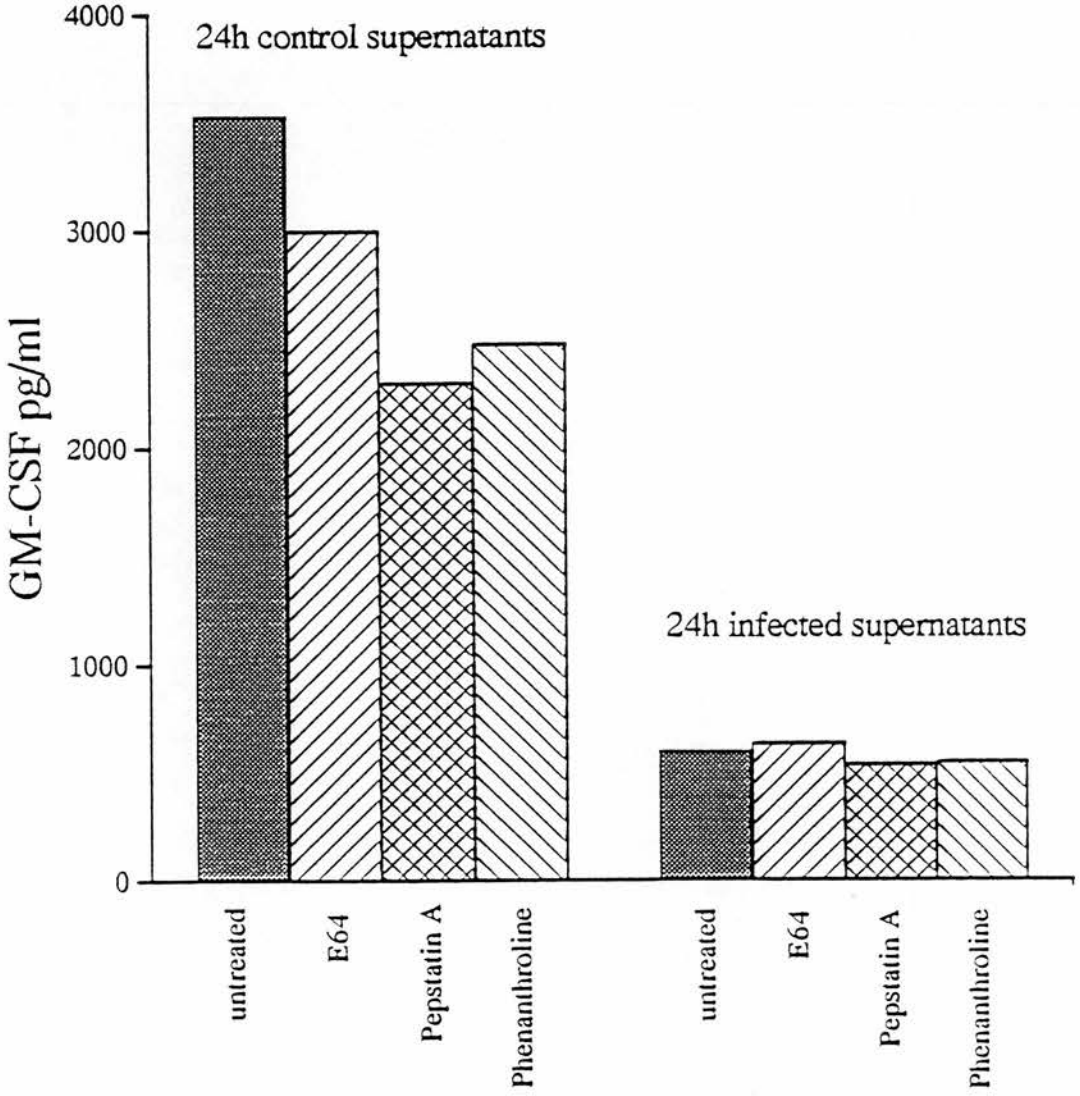
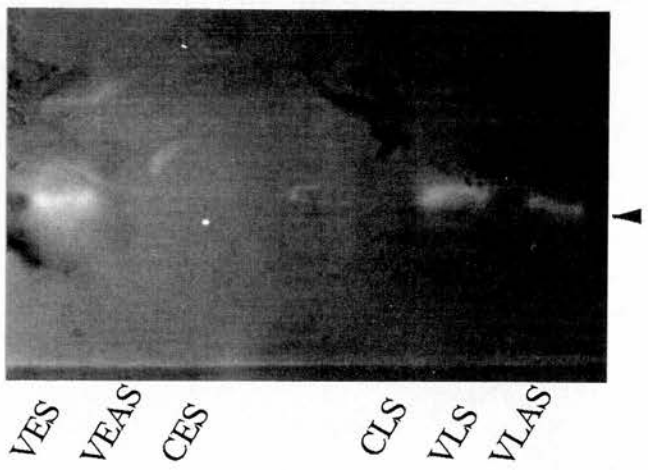



**Figure 5.14** *The effect of protease inhibitors on the clearance of recombinant ovine GM-CSF by culture supernatants collected from control and orf 11 virus infected keratinocytes (at 24h post-infection).*

asocasein gel at a molecular weight of approximately 100-130 KD (not shown). Analysis of the supernatants showed that the early collection (2h post-infection) from the virally infected cells contained proteolytic activity at a molecular weight of approximately 28 KD in the asocasein gel (Figure 5.15). This proteolytic activity was not observed in the supernatants from the control cells or the early collection of infected cells which were incubated with actinomycin D. The late collection (9h post-infection) of infected supernatants also contained this band of proteolytic activity, at 28 KD which was also present in the infected supernatants containing actinomycin D. No bands were present in the late, control supernatants.




CES= control early sample
VES= virus early sample
VEAS= virus early + actinomycin D
CLS= control late sample
VLS= virus late sample
VLAS= virus late sample + actinomycin D

**Figure 5.15** *Asocasein gel analysis of keratinocyte proteolytic activity. Early and late collections of the supernatants from control and orf 11 virus infected keratinocytes with and without actinomycin D were analysed for their proteolytic activity which could be detected by a clear band (of approximately 100-130 kD) within the gel.*

## 5.3. DISCUSSION

Ovine keratinocytes were screened for their ability to produce the cytokines IL-1β, IL-3, IL-8, TNFα, GM-CSF and interferons (α, β and γ). The production of cytokine protein was measured in a number of ELISA and bioassays specifically developed for the detection of ovine cytokines. As cytokines exert their effects at low concentrations, sometimes below those detectable by bio- or ELISA assays. The RT-PCR technique was utilized to measure the production of cytokine mRNA. The strategy used in the present study provided only semi-quantitative results, which enabled the assessment of relative changes in mRNA synthesis. Although the cDNA for each of the cytokines was prepared from the same concentration of cellular RNA, signal strength is dependent on the efficiency of the primers used in the RT-PCR and of the probes used in hybridisation. Thus the signal strength of each cytokine mRNA can only be compared to itself.

### 5.3.1. IL-1β, TNFα, Interferons and IL-3

The anti-viral properties and the increased production by keratinocytes of the cytokines IL-1β, TNFα and interferons in response to HSV-1 (Sprecher and Becker, 1992; Torseth *et al.*, 1987) would indicate a potential role for these cytokines in the keratinocyte response to other viral infections. However this study failed to detect the induction of any of these cytokines following orf virus infection. Similarly these cytokines were not induced by stimuli such as TPA or UV irradiation. It is possible that ovine keratinocytes, unlike their human and murine counterparts lack the ability to produce these cytokines, although this seems unlikely. The bioassays were sensitive only to 30,000 pg/ml for TNFα, and approximately 50 pg/ml for interferon and so it may be that the methods of detection used lack the sensitivity required for low cytokine production, or it is possible that the stimuli of TPA or UVB irradiation, which were not optimised, may not have been adequate for the induction of these cytokines.

All that can be concluded is that if these cytokines are being produced, then it must be at a level below the sensitivity of the detection methods used. Recently, the induction of human keratinocyte TNFα by UVB irradiation, first described by Kock *et al.*, (1990) has proved difficult to reproduce (Dr Mary Norval, personal communication) indicating that variations in experimental procedures may affect the induction of cytokine production.

Keratinocytes of several species, as well as transformed keratinocytes and carcinoma cell lines, have been shown to spontaneously release molecules with IL-1 activities, whose production is significantly increased in response to mitogens and UVB irradiation (Kupper *et al.*, 1986). Recently, it has been demonstrated that although cultured human and murine keratinocytes express mRNA encoding IL-1α and IL-1β, all IL-1 biological activity that is produced by keratinocytes *in vitro* and in the epidermis is due to IL-1α (Kupper *et al.*, 1986, 1988). Keratinocytes *in vitro* also express high affinity receptors for IL-1 (Kupper *et al.*, 1988). Thus keratinocytes can potentially respond in an autocrine fashion, the significance of this *in vivo* will be discussed in more detail below. Ovine IL-1α was not investigated in this study and it may be the type of IL-1 preferentially produced by ovine keratinocytes, which would explain the absence of IL-1β. The production of IL-1α by ovine keratinocytes in response to orf virus infection would be interesting to study and will require the development of an ELISA or bioassay, as currently there is no method of measuring ovine IL-1α production.

The anti-viral activities of the interferons led to their discovery (Wheelock 1965). Although IFNγ production has not been demonstrated for keratinocytes, keratinocytes upon infection with HSV-1 release cytokines with anti-viral activity (Schnipper *et al.*, 1984) which was demonstrated to belong to the IFNα group (Yaar *et al.*, 1988). However at present it has not been formally proven whether keratinocytes are capable of producing IFNα or IFNβ. The interferon assay that was used should detect any interferon activity (α, β, or γ) present within the culture supernatants, but it may not have been sensitive enough to detect low amounts of IFN activity. IFNγ mRNA only

was studied and so a more detailed analysis of keratinocyte IFN mRNA using primers and probes for IFNα and IFNβ is required to confirm the lack of IFN production by orf virus infected keratinocytes. The selection of the primers from the wide range of possible interferons produced will be the main problem for mRNA analysis.

The production of IL-3 by keratinocytes is less well defined, although IL-3 like activity has been found in murine neonatal keratinocytes and the transformed cell line Pam 212 (Luger *et al.*, 1989). Production of IL-3 has not been demonstrated in human keratinocytes. The study of ovine keratinocytes would indicate that, at the level of mRNA, IL-3 may be produced in response to orf virus infection, but the inconsistent mRNA patterns that were observed highlight the need of a bio- or ELISA assay to identify IL-3 activity within culture supernatants.

### 5.3.2. IL-8

Interleukin 8 (IL-8) is a newly identified potent chemotactic cytokine for neutrophils (Matsushima *et al.*, 1992) and T lymphocytes (Larsen *et al.*, 1989). IL-8 has been shown to be synthesised by a number of different cell types including monocytes/macrophages, dermal fibroblasts and keratinocytes (Zachariae *et al.*, 1992). The production of IL-8 is generally not constitutive but can be induced by several stimulants including mitogens and viruses (Matsushima *et al.*, 1992). This study confirmed that IL-8 could be produced by ovine keratinocytes. Initially it appeared that IL-8 was constitutively produced by resting keratinocytes. However the production of such a high level of IL-8 was found to be in response to unknown stimuli within the FCS included in the culture medium. IL-8 was similarly induced by the stimuli of TPA and in response to orf virus infection. IL-8 was induced early in infection by both strains of orf virus (Scabby Mouth and orf 11) in a virtually identical manner. It was only at 24h that a difference became apparent. A decrease in IL-8 production was observed for the Scabby Mouth infected cells whereas the level of IL-8 in the orf 11 infected cells remained high. This is likely to be due to the more rapid cytopathic effect caused by Scabby Mouth. The induction of IL-8 *in vitro* could be tentatively related to the *in vivo* orf lesion, as IL-8 might induce the infiltration of T

lymphocytes and neutrophils into the skin observed in response to orf virus infection. This will be discussed in chapter 6.

### 5.3.3. GM-CSF

GM-CSF is a glycoprotein that was first identified by its ability to promote differentiation of haematopoietic progenitor cells to mature granulocytes and macrophages (Metcalf, 1985). A variety of cells types including mononuclear phagocytes (Chevernick *et al.*, 1972, Golde *et al.*, 1972), T lymphocytes (Cline *et al.*, 1974, Ruscetti *et al.*, 1975), fibroblasts (Guez *et al.*, 1973) and keratinocytes (Kupper *et al.*, 1988) are known to produce GM-CSF. Synthesis of GM-CSF by keratinocytes can be upregulated by tumour promoters, other cytokines and UV irradiation (Nozaki *et al.*, 1991).

The present study showed that ovine keratinocytes can be stimulated to produce GM-CSF. In resting cultures, the constitutive production of GM-CSF was, like IL-8, found to be as a result of the unknown stimuli contained within the FCS of the culture medium. The presence of TPA did not appear to induce the production of GM-CSF. UVB irradiation increased GM-CSF production by keratinocytes, but this induction was found to be dependent on wavelength and dose. Broad band UVB light (270nm-320nm) but not narrow band UVB (312nm) induced GM-CSF mRNA, confirming recent studies (Dr Mary Norval, personal communication) where narrow band UVB light was found to be ineffective at stimulating cytokine production. The high dose of UVB increased GM-CSF mRNA but not the production of protein which may be due to an inhibition of translation or the death of the cells before the message can be translated. The low dose of UVB did not damage the cells, and the high level of mRNA was reflected in a high level of GM-CSF protein detected in the supernatants. Nozaki *et al.*, (1991) showed that the induction of GM-CSF was mediated by UV induced IL-1$\alpha$. It is possible therefore that the induction of GM-CSF that was observed in this study is mediated by a similar mechanism and highlights the need to investigate IL-1$\alpha$ production by ovine keratinocytes in future studies.

In the present study, we have shown that ovine keratinocytes possess the ability to produce GM-CSF, which can be enhanced by UVB. However on infection with orf virus, GM-CSF production by keratinocytes was not induced and instead appeared to be inhibited. The inhibition of GM-CSF production appeared to be a post-transcriptional effect as GM-CSF mRNA in the presence of high serum was detected in both the control and infected keratinocytes at equal levels. The amount of GM-CSF protein that was subsequently released into the supernatants by the infected cells was however dramatically reduced compared with the control cells. Even without the stimulus of high serum, it appeared that orf virus may induce GM-CSF mRNA production (2h-4h post-infection) and yet this was not reflected in the secretion of GM-CSF into the supernatants. Thus orf virus inhibits the production of GM-CSF at a point after transcription.

Theoretically orf virus could interfere with the production of GM-CSF in three main ways;

1. The process of translation of GM-CSF mRNA into protein could be inhibited by the virus.

2. Post-translational modifications (such as glycosylation) required for the transport of GM-CSF out of the cell could be prevented, thereby trapping GM-CSF protein within the cell.

3. The virally infected cells may produce a counteractive protein that is released into the supernatants that alters the GM-CSF protein produced by the cells, preventing its detection by ELISA.

All three of these mechanisms could be employed by orf virus, but in this study the last possibility was examined in more detail.

A simple experiment, whereby a known concentration of exogenous GM-CSF was added to supernatants collected from orf virus infected cells revealed the presence of a factor or factors that interfered with the detection of GM-CSF. This effect was

specific for the supernatants from infected cells and, although GM-CSF inhibition occurred within the first 10h post-infection, it was most marked at 12h and 24h post-infection. Whatever the nature of the inhibitory factor(s), it prevents the recognition of one or both of the epitopes of GM-CSF by the antibodies in the ELISA. The two antibodies used in the ELISA are neutralising antibodies (Entrican *et al.*, 1994) and so this suggests that the inhibitory factors also block the biological activity of GM-CSF which would need to be confirmed in a bioassay.

Although control and orf virus infected keratinocytes were identified as containing intracellular protease activity this activity was not released into either culture supernatants. In addition however separate proteolytic activity, of a different size was observed in orf infected supernatants only, at both early and late timepoints post-infection. The production of this protease by the virally infected cells was inhibited at the early timepoint post-infection by the presence of actinomycin,which inhibits new mRNA synthesis and hence viral protein synthesis, indicating the possible viral nature of the protein. The effect of the actinomycin was overriden later in the infection as the virally induced protease could be detected in the supernatants of late collections despite the presence of actinomycin. The nature and specificity of the protease was not identified. Whilst the infected cells were shown to produce protease activity the addition of proteolytic inhibitors to the supernatants did not prevent the inhibition of GM-CSF. Although, this proteolytic activity is not involved in the inhibition of GM-CSF it would be interesting to characterise fully this virally induced proteolytic activity and to identify its role in an orf virus infection.

The GM-CSF clearance was, however, affected by the process of heating. The ability of the 12h infected supernatant to interfere with the added exogenous GM-CSF was completely abolished by heating. Although the inhibitory effect of the 24h infected supernatant was affected, it was only partially reduced when compared to the un-heated supernatant. These results, along with the fact that the inhibition of GM-CSF does not appear to be mediated by a protease, might indictate the production of a protein released into the supernatants of the virally infected cells that binds specifically to GM-CSF. The process of heating may alter the confirmation of this protein which

may prevent it from binding to GM-CSF and so preventing the inhibition. Heating did not completely reverse the inhibition of GM-CSF for the 24h infected supernatant, and it is possible that this is because of the large quantity of the inhibitory protein present in the culture supernatant, whose activity may not be completely abolished by heating.

The production of a soluble GM-CSF receptor analogue in orf virus infected keratinocytes is an interesting possibility, as recently other members of the pox virus family have been shown to produce a number of cytokine receptor analogues for IL-1, TNF and interferon. These viral receptor analogues are postulated to interfere with the host response to pox viruses by binding the cytokines in the exracellular space and preventing them from reaching their target receptors on the surface of cells

Future studies will determine whether orf virus does have the ability to code for such a receptor analogue for GM-CSF.

### 5.3.4. Conclusions

It emerged from this study that orf virus induces IL-8 production but inhibits the production of GM-CSF in cultures of infected ovine keratinocytes. It would appear that the inhibition of GM-CSF is a specific effect that occurs early in infection and does not affect GM-CSF transcription. The inhibition of GM-CSF is mediated, at least in part, by factor(s) released into the supernatant from the infected cells. The exact nature of the inhibition was not identified but the results from this study might indicate the possession by orf virus of a gene coding for a soluble GM-CSF receptor analogue, which binds to secreted GM-CSF. The failure to detect IL-1, TNF$\alpha$ and IFNs, cytokines that would be expected to be produced by keratinocytes in response to orf virus infection, raises the possibility that orf virus also inhibits their production. However, as these cytokines were not demonstrated for control or stimulated keratinocytes, it is unclear if they are inhibited by orf virus. The ability of the virus to interfere with specific cytokines emphasises the importance of these mediators in the host response to virus infections. The significance of these *in vitro* results as applied

to the *in vivo* observations of an orf virus infection will be discussed in detail in chapter 6.

# CHAPTER 6

## GENERAL DISCUSSION

The ability of orf virus to re-infect its host despite the generation of a specific humoral response and the localised nature of the infection, led workers to the conclusion that local, cellular immunity is probably the major factor in recovery and protection from orf virus infection (Robinson and Balassu, 1981). The main aim of this thesis was to characterise the ovine skin response to orf virus infection.

Studies carried out previously at the Moredun Research Institute examined the local cellular immune response to orf virus and demonstrated an influx of inflammatory cells, involving a large number of neutrophils and T lymphocytes, into the dermis underlying the infected epidermis (Jenkinson *et al.*, 1990a). The cutaneous defence mechanisms after primary and secondary challenge with orf virus were concluded to be essentially the same, even though a secondary infection is generally milder and resolves faster than a primary infection (Jenkinson *et al.*, 1990b). Further studies of the secondary orf lesion revealed the formation of a dense network of MHC class $II^+$ dendritic cells directly beneath the lesion (Jenkinson *et al.*, 1991). These cells were postulated to have an important barrier function preventing the downward progression of the virus into the underlying dermis.

The experiments described in this thesis were therefore designed to extend our knowledge of the local, cellular response to orf virus by characterising in more detail the dendritic cell network of both the primary and secondary orf virus lesions. These experiments were aimed at determining the relationship of the accumulating dendritic cells with the dendritic cells normally resident within the skin, the kinetics of the response and how or from where the network of dendritic cells originates. Studies of cytokine production by ovine keratinocytes infected *in vitro* with orf virus were also undertaken to determine the ability of the virus to induce or suppress a range of cytokines, which may play an important role in the local immune response to orf virus infection *in vivo*.

This final chapter will attempt to bring together the main findings of the thesis and discuss them in relation to what is known about orf virus and other viral infections of the skin.

## Orf virus induction and inhibition of keratinocyte cytokine production

Orf virus enters through abrasions in the skin where it replicates in the newly regenerating epidermal keratinocytes (Jenkinson *et al.*, 1990a). Keratinocytes, which constitute the bulk of the epidermal cell population, have been implicated as playing an important role in the immunological control of viral infections by their ability to produce a wide range of cytokines that can act locally to modulate LC function and induce inflammation (Sprecher and Becker, 1992). Thus it was proposed that the infection of keratinocytes with orf virus may stimulate the release of cytokine signals that initiate the influx of inflammatory and dendritic cells into the skin and prevent the spread of the virus.

Normal ovine keratinocytes cultured from the follicular epithelium of plucked sheep hairs (chapter 4) were shown to produce the neutrophil and T cell chemoattractant IL-8. The *in vitro* production of IL-8 was increased when the keratinocytes were stimulated with TPA or infected with orf virus (chapter 5). It could be envisaged therefore, that, *in vivo,* IL-8 is produced initially in response to the epidermal damage caused by scarification and subsequently in response to orf virus replication and results in the biphasic influx of large numbers of neutrophils and lymphocytes that are observed in both primary and secondary orf lesions. Increased numbers of neutrophils are observed in the afferent lymph following scarification but not in response to viral replication (Yirrell *et al.*, 1991).The increased production of IL-8 observed in response to orf virus infection could be responsible for trapping the neutrophils at the site of infection and preventing their migration into the lymph.

Other cytokines, demonstrated to be produced by human and murine keratinocytes with anti-viral activities such as IL-1, TNF, and IFNs that would be expected to play a role in opposing orf virus progression were not demonstrated to be produced by orf virus infected keratinocytes *in vitro*. In addition to the absence of these cytokines, orf virus was shown to specifically interfere with the production of GM-CSF by the cultured ovine keratinocytes. The inhibition of GM-CSF production by orf virus

infected keratinocytes appeared to be mediated at least in part by a soluble factor or factors released into the culture supernatants. This inhibition was unaffected by the addition of specific protease inhibitors, indicating that proteolytic degradation of GM-CSF by the release of viral proteases is unlikely to be responsible for the effect. One possiblity is that the orf virus infected keratinocytes release a soluble receptor analogue protein into the supernatants that interferes with any GM-CSF present. The inhibition of GM-CSF by a soluble receptor produced by orf infected cells is a very interesting possibility as recently a number of virally encoded receptor homologues of cellular receptors have been identified for other members of the pox virus family.

## Pox virus cytokine receptor analogues

The first cytokine receptor homologue to be identified was a TNFR in myxoma virus, Shope fibroma virus and malignant rabbit virus (Smith *et al.*, 1991, Upton *et al.*, 1991, see chapter 1). Since then receptor homologues for IFNγ have been found in Myxoma virus, Shope fibroma virus and vaccinia virus (Upton *et al.*, 1992) and an IL-1β receptor analogue has also been discovered in vaccinia virus (Alcami and Smith, 1992). These receptor analogues were originally identified in the viral genomes by computer homology scores with their cellular counterparts. Not all of the homologues identified, however, result in the expression of active proteins. Vaccinia virus lacks a functional TNFR as the gene sequence encoding it is fragmented by frame shifts and stop codons. Complete receptors for TNF (myxoma virus and Shope fibroma virus), IL-1β (vaccinia virus) and IFNγ (myxoma virus) have been demonstrated to be secreted either by virally infected cells or by cells transfected with the relevant viral gene, and they have the ablity to bind the specific cytokine. TNF, IL-1β and IFNγ are all molecules with important and overlapping functions in the inflammatory immune response to viral infections. The cytokine receptor analogues produced to counteract them are secreted in a soluble form by infected cells with the trans-membrane anchor sequence and cytoplasmic tails absent. These soluble receptors are then believed to compete with the cellular receptors for the free cytokines, effectively intercepting and short circuiting the anti-viral immune response pathways dependent on the cognate

cytokine. The ability of the pox viruses to interfere with TNF, IL-1β and IFN suggests a role for these cytokines in combating pox virus infections. The importance of IL-1β in vaccinia virus infection is highlighted by the nature of the specificity of the inhibition - the IL-1 receptor analogue will bind IL-1β but not IL-1α (Alcami and Smith, 1992). In addition vaccinia virus expresses a serine protease inhibitor (serpin) which inhibits, *in vitro,* the conversion of the IL-1β precursor form into the secreted and active form (Ray *et al.,* 1992).Thus, if orf virus specifically inhibits GM-CSF, then it is possible that GM-CSF is important in combating orf virus infections; this will be discussed in more detail below.

A receptor homologue for GM-CSF has not yet been described for the pox virus family of 'viroreceptors' (Upton *et al.,* 1991). The present study indicates only that there is inhibitory activity for GM-CSF in the culture supernatants of orf virus infected cells. The specificity of this inhibition and the nature of the factors involved need to be identified and a much more detailed study of the orf virus genome will be required to determine whether there is a gene encoding a GM-CSFR homologue. The orf virus genome is for the most part uncharacterised. Sequencing of selected regions has shown that there is a significant degree of conservation of structure and arrangement of genes between orf virus and vaccinia virus. The latter virus has been completely sequenced (Fleming *et al.,* 1993 and Goebbel *et al.,* 1990). The similar locations and orientations of homologous orf virus and vaccinia virus genes on their respective genome maps has allowed the tentative alignment of the two genomes (Lyttle *et al.,* 1994). Despite the regions of conservation, significant differences between the two viral genomes were discovered (Fraser *et al.,* 1990, Fleming *et al.,* 1993). The genes closest to the termini of the pox virus genomes appear to be the regions of interest as they encode a number of different virulence factors, including the cytokine receptor homologues. The region of the vaccinia virus genome encoding the identified cytokine receptor homologues, however, lies outwith the aligned orf virus genome. As there are substantial areas of the orf virus genome between the overlapping regions that have not been characterised, the possibility of the possession of a number of uncharacterised virulence factors by orf virus cannot be ruled out.

The complete absence of IL-1β, TNF and IFNs from control, stimulated and infected keratinocyte cultures is puzzling. The possible reasons for this are discussed individually in chapter 5. The results from chapter 4 showed that host cell protein synthesis is increased during the first 12h after orf virus infection, therefore dispelling the possibility that the absence of cytokine production by the infected cells is due to a non-specific shutdown of all host cell metabolic activity. Although the cytokine detection systems may have lacked the sensitivity required to detect these cytokines, another consideration is that orf virus also interferes with the production of a number of cytokines including IL-1β, TNF and IFN. This could be through the production of specific cytokine receptors or by other unknown mechanisms. The development of assay systems with increased sensitivity for ovine cytokines and the complete molecular analysis of the orf virus genome will clarify the ability of the virus to interfere with the host immune response.

The possession of virulence factors that enable the evasion of specific immune effector mechanisms would be beneficial to the virus, possibly leading to the prolongation of the infection, ineffective clearance of the virus from the site or by increasing the chances of developing lesions again on re-infection. Thus, if the inhibition of GM-CSF by orf virus is a specific mechanism employed by the virus *in vivo,* what is the significance of this for the host and can it be related to the incomplete immunity to orf virus infections ?

## The significance of GM-CSF inhibition in the host response to orf virus infection

The cutaneous inflammatory response that is observed after a secondary orf infection was believed to differ from a primary infection only by the timescale and severity of the reaction (Jenkinson *et al.*, 1990b). Whilst a secondary infection results in an accelerated and milder response, orf virus is still able to replicate successfully within the epidermis and infectious virus is shed into the environment. Thus, the cellular immunity generated locally in the skin is not fully protective.

The MHC class II$^+$ dendritic cells that gather in response to orf virus infection represent a population of dendritic cells separate from the epidermal LCs of normal ovine skin (Jenkinson *et al.*, 1991). Further characterisation of both primary and secondary orf lesions identified these cells as a population of CD1$^-$ dendritic cells, sharing the phenotype of a small subpopulation of dendritic cells found in the dermis of normal ovine skin (chapter 3). A second marker, factor XIIIa, expressed by a small number of the resident CD1$^-$ dermal dendritic cells revealed significant differences in the phenotype of the dendritic cells accumulating at a primary orf lesion compared with a secondary orf lesion. Factor XIIIa$^+$ dendritic cells were present in much higher numbers and persisted for much longer in the primary orf lesion compared with the small numbers of factor XIIIa$^+$ dendritic cells transiently observed in the secondary orf lesion. Recent research has implicated factor XIIIa$^+$ dendritic cells in playing a central role in wound repair and the skin immune response (chapter 3). Factor XIIIa is a marker that is also found on the surface of monocytes and is retained during their differentiation into macrophages (Adany *et al.*, 1985). The relationship between dendritic cells and the monocyte-macrophage lineage remains highly controversial. One school of thought believes that the dendritic cells arise from a separate haematopoietic cell lineage distinct from macrophages, but which share a common progenitor cell (Steinman, 1983) whereas another proposes that monocytes can differentiate into dendritic cells as oppposed to macrophages when appropriately stimulated (Peters *et al.*, 1991). Jenkinson *et al.*, (1991) failed to detect a high number of dendritic cells circulating in the blood that could account for the formation of such a dense network of cells and proposed that these cells may circulate in a precursor form which then differentiate at the site of infection. Thus it could be envisaged that the factor XIIIa$^+$ dendritic cells that accumulate in a primary infection represent an immature population of cells that have retained the macrophage marker factor XIIIa and are dependent upon the local production of cytokines to fully differentiate into mature dendritic cells.

Factor XIIIa$^+$ dendritic cells have been associated with immunosuppression and are involved in the secondary verrucous lesions that develop in HIV$^+$ individuals that are

infected with herpes simplex virus or human papilloma virus. The presence of these factor XIIIa$^+$ dendritic cells in the lesions of these patients could be related to alterations in the local cytokine profile in the skin due to the HIV induced immunosupression.

Recently, the cytokines GM-CSF and TNFα have been shown to support the *in vitro* development of dendritic cells from haematopoietic progenitor cells present in blood and bone marrow (Caux *et al.*, 1992, Reid *et al.*, 1992). These cytokines also support the survival and or proliferation of isolated murine epidermal LCs and influence their development into potent T-cell stimulatory accessory cells (Heufler *et al.*, 1988, Koch *et al.*, 1990). Veiled dendritic cells, isolated from ovine afferent lymph, survive *in vitro* in the presence of TNFα and increase in number if GM-CSF and TNFα are added in combination to the cultures (Haig *et al.*, 1994). It would appear therefore, that GM-CSF and TNFα play an important role in the maturation and survival of dendritic cells *in vitro*.

*In vivo,* the maturation of precursor cells into mature dendritic cells may be dependent therefore, upon the local production of the cytokines GM-CSF and TNFα. Thus, if orf virus inhibits the release of GM-CSF and possibly TNFα by keratinocytes *in vivo* then the dendritic cell precursors accumulating in response to a primary orf virus infection may be prevented from differentiating, resulting in a network of phenotypically and functionally immature dendritic cells. The factor XIIIa$^+$ dendritic cells of the primary orf lesion represent only half of the dendritic network. The factor XIIIa$^-$ cells comprising the other half may have been able to mature in the presence of low amounts of GM-CSF and TNFα produced by other cells present. T cells, which are potent producers of GM-CSF (Nozaki *et al.*, 1991), and factor XIIIa$^+$ dendritic cells which also produce TNFα and may represent an alternative source of these cytokines. The production of these cytokines in a secondary infection, where there is an accelerated response may be sufficient to compensate for the lack of the keratinocyte cytokine source and induce the maturation of the accumulating dendritic cells as they enter the dermis, resulting in the transient expression of factor XIIIa that is observed. It is also possible that the factor XIIIa$^+$ dendritic cells of the primary orf

lesion return to the peripheral circulation after the resolution of infection, where they lose the surface marker factor XIIIa. On a secondary infection they may migrate preferentially into the skin to give an accelerated response and a milder infection.

Recent studies at the Moredun Research Institute have shown that intradermal injections of GM-CSF and TNF$\alpha$ into ovine skin induces an accumulation of MHC class II$^+$ dendritic cells similar to that observed in response to an orf virus infection. These dendritic cells lack detectable levels of both CD1 and factor XIIIa (Haig *et al.*, 1994). It was concluded that GM-CSF in particular is involved in an *in vivo* pathway that induces the accumulation of predominantly (but not only) MHC class II$^+$/CD1$^-$ dendritic cells. These results support the significance of GM-CSF *in vivo,* in generating a network MHC class II$^+$ dendritic cells that lack the expression of factor XIIIa. *In vitro* however, neither fresh nor cultured ovine afferent lymph dendritic cells in the presence of TNF$\alpha$ or GM-CSF, expressed the marker factor XIIIa. Therefore more detailed studies are required to determine whether the expression of the dendritic cell markers can be regulated by specific cytokines and whether the expression of the markers reflect functional differences.

The origins of the accumulating dendritic cells of primary and secondary infections were not established. Although both CD1$^-$/factor XIIIa$^+$ and CD1$^-$/factor XIIIa$^-$ dendritic cells represent small subpopulations of dermal dendritic cells found in normal skin the results showed that it is unlikely that these cells proliferate locally to form the network of cells seen in response to infection. The general lack of cellular proliferation in the dermis of primary and to a greater extent secondary orf lesions would support the theory that the cells infiltrate into the skin from the blood. The origin and relationship of these cells with the monocyte-macrophage lineage remains to be determined. The network of MHC class II$^+$ dendritic cells did not share surface markers that were found on ovine macrophages, regardless of their expression of factor XIIIa. Thus, if the dendritic cells represent a population of macrophage-related precursor cells, then they have lost the expression of a number of macrophage surface markers and may represent an indeterminate stage of differentiation.

## The proliferative nature of the primary orf lesion

Studies of the reaction of abraded and scarified ovine skin to challenge by orf virus have demonstrated that viral replication begins after initial repair of the epidermis and dermis following the trauma. During this first 48h of repair, viral antigen cannot be detected and this period is known as the eclipse phase (Osman, 1976). Orf virus appears to require the proliferation of the epidermal cells before it can replicate, as the viral growth cycle begins during the differentiation of the new epidermis at a stage which has still to be identified (Jenkinson *et al.*, 1990c). *In vitro* studies of orf virus replication also indicated the requirement for cellular proliferation as young and rapidly dividing cells were found to be more sensitive to viral infection than older cultures (Nagington *et al.*, 1968). *In vivo*, the proliferating cells of the newly regenerating epidermis at 48h post-infection were highlighted by their staining with the antibody PCNA (chapter 3).

At this stage, there was no difference between a primary and a secondary infection. These proliferating cells were able to support viral replication with the appearance of viral antigen, detected at 3-4 days post-infection. The infection spreads laterally and uniformly along the new epidermis encompassing the entire depth of the epidermis except for the *stratum basale* (chapter 3). In a secondary infection the proliferation of the epidermis, as detected by PCNA, decreased, coinciding with the disappearance of the viral antigen. The primary orf lesion however, is highly proliferative and this is reflected in the persistence of the virus with viral antigen still detected on day 22 post-infection. The proliferative nature of naturally occurring orf virus infections and the hypertrophic appearance of the regenerating epidermis have emphasised the effect of orf virus on epidermal growth. Vaccinia virus encodes an epidermal growth factor (EGF) related protein (Brown *et al.*, 1985) that is thought to mediate binding of the virus to skin cells expressing the receptor or to stimulate the growth of uninfected cells and promote the spread of infection. Extensive molecular analysis of the orf virus genome failed to locate an EGF gene analogous to that found in vaccinia (Lyttle *et*

*al.*, 1994). However a gene encoding a polypeptide with homology to mammalian vascular endothelial growth factor (VEGF) was identified in the genome of orf virus (Lyttle *et al.*, 1994). The VEGF of orf virus could be directly responsible for the extensive vascular endothelial proliferation that characterises human orf virus lesions. However, this is not a feature of ovine orf lesions. How the VEGF benefits the virus is unknown, but it is thought that it could act by increasing the flow of metabolites essential to the virus or by inducing epidermal proliferation indirectly. VEGF is highly specific for endothelial cells but Yirrell *et al.*, (1994) postulated that orf virus VEGF may induce epidermal instead of endothelial proliferation in sheep, thereby providing the virus with a fresh source of proliferating cells in which to replicate. The reduced epidermal proliferation that is observed in a secondary orf virus infection may occur as a result of the faster clearance of the virus from the skin, resulting in a lower level of VEGF released. However, it may be that the two features of the primary orf lesion that differentiate it from a secondary orf lesion, factor XIIIa$^+$ dendritic cells and extensive epidermal proliferation, are related.

The factor XIIIa$^+$ dendritic cells of the primary orf lesion were observed to be clustered directly beneath the proliferating epidermis. Such a close association of factor XIIIa$^+$ dendritic cells has also been described in the highly proliferative psoriasis lesions in humans (Cerio *et al.*, 1989). The combined *in vitro* and *in vivo* studies (Nickoloff *et al.*, 1991, Barker *et al.*, 1990, 1991 and Nickoloff *et al.*, 1986) clearly delineate important, spatially co-ordinated cytokine-controlled reactions between the TNF producing dermal dendritic cells and the overlying keratinocytes which have increased IL-8, ICAM-1 and TGF$\alpha$ expression. The increased TGF$\alpha$ is known to induce keratinocyte proliferation which would account for the hyperplasia of keratinocytes in psoriatic lesions. The similarity of the primary orf lesion with the psoriatic lesions of man could be related to the presence of the factor XIIIa$^+$ dendritic cells.

Factor XIIIa$^+$ dendritic cells are known to be involved in the initial stages of healing, possibly by stimulating the proliferation of the epidermal cells and thereby knitting the edges of the wound together. In orf virus infected skin these proliferating epidermal

cells support orf virus replication. In a primary orf lesion, the factor XIIIa$^+$ dendritic cells that accumulate may produce TNF$\alpha$ which would result in the production of TGF$\alpha$ by the keratinocytes and the extensive proliferation of the epidermis. The continued proliferation of the epidermal keratinocytes would provide orf virus with a fresh source of cells to infect and may increase the spread of the virus along the epidermis resulting in a more prolonged and severe infection. The increased spread of the virus and the formation of a network of immature dendritic cells could account for the more severe primary orf virus infection and also prevent the development of complete immunity, thus increasing the chances of developing lesions again on re-infection with orf virus.

As the function of both populations of CD1$^-$/factor XIIIa$^+$ and CD1$^-$/factor XIIIa$^-$ dermal dendritic cells have not yet been determined the sequence of the events at primary and secondary orf lesions, described above, is merely speculative. More detailed studies will be required to determine the role of these cells in the response to orf virus infections and the ability of specific cytokines to regulate the expression of their surface markers.

## In summary

Orf virus represents one of a group of viruses including vaccinia virus, HSV and HPV that infect the skin, replicating within the keratinocyte cell population. The skin lesions caused by each of these viruses are all different and the orf lesion that develops in humans is also significantly different from the orf lesion that develops in sheep. Although the local, cellular immune response is important in the control of each of these infections, the differences observed in the lesions will reflect a combination of the involvement of different components of the immune system and the ability of the virus concerned to replicate and alter or evade these responses. The importance of cellular immunity for the control of infections with vaccinia virus, HPV and HSV has been emphasised by the severity and persistence of the infections that develop when various components of the cellular immune system are depleted (chapter 1). Thus studies of orf virus infections in sheep that are immunocompromised in some way may

increase our understanding of the importance of local, cellular immune effector mechanisms in the host response to orf virus infections.

In conclusion, there is still much work to be done before the interaction of orf virus with the local, cellular immune system of the skin can be fully elucidated. The results presented in this thesis indicate a difference in the dendritic cell response of primary and secondary orf virus infections that may be related to the differences in the time course and severity of the lesions. These results also support the increasing recognition of the importance of dermal dendritic cells in the skin response to infections. In addition, the ability of orf virus to interfere with the production of the cytokine GM-CSF by keratinocytes was reported. This indicates the possible possession by orf virus of virulence factors which may enable the evasion of specific effector mechanisms and be related to the incomplete immunity generated after an orf virus infection. This thesis has hopefully provided a basis for future work which will further characterise the ability of orf virus to interfere with the host response. Characterisation of these putative virulence factors would add to our knowledge of the components of the immune system important in combatting orf virus infections, which may lead to more effective treatment and the development of improved vaccines against orf virus.

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# APPENDIX

## WORK SUBMITTED FOR PUBLICATION

## Publication

A copy of the manuscript submitted for publication is found below and was derived from the work contained within this thesis. Photographs and graphs are not included but can be found within Chapter 3.

Lear, A; Hutchison G; Reid H.W; Norval, M and Haig D.M (1994). Phenotypic characterisation of the dendritic cells accumulating in the ovine dermis following primary and secondary orf virus infections. Submitted for publication to Veterinary Dermatology.

**Phenotypic characterisation of the dendritic cells accumulating in the ovine dermis following primary and secondary orf virus infections.**

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Short running heading: Orf virus infections and dendritic cells

## ABSTRACT

Four populations of MHC-class II$^+$ dendritic cells were characterised within normal ovine skin. The MHC-class II$^+$ dendritic cell accumulation observed in response to primary and secondary orf virus infections of sheep comprised two of the populations that normally reside in the ovine dermis. These accumulating dendritic cells lacked the CD1 antigen and a proportion expressed factor XIIIa. The highest number of factor XIIIa$^+$ dendritic cells accumulated in a primary infection, with small numbers appearing only transiently in a secondary infection.

Only a few of the dendritic cells underwent proliferation in a primary orf infection, suggesting that the network forms largely by an influx of cells into the dermis from the blood. The dendritic cells were not recognised by a panel of monoclonal antibodies which labelled macrophages and their origin remains unknown.

# INTRODUCTION

Orf, an eruptive and localised skin disease of sheep and goats, is caused by a parapox DNA virus. The disease which can also affect man, is endemic globally in sheep, being most prevalent in young lambs and their nursing ewes (1). It is an acute infection in which the virus enters through abrasions in the skin and replicates within regenerating epidermal keratinocytes (2). The host response to orf virus infection involves an accumulation of a large number of neutrophils and lymphocytes adjacent to and underlying the infected epidermis (3). A characteristic feature of the orf lesion is a dense accumulation of MHC class $II^+$ dendritic cells which have been hypothesised to form a barrier to viral invasion and a template for subsequent epidermal repair (4). Jenkinson et al (4) showed that these accumulating dendritic cells do not express acetylcholinesterase (AchE), a marker expressed by normal ovine epidermal Langerhan's cells but not by dermal dendritic cells.

This study sought to determine, firstly, whether the dendritic cells accumulating adjacent to or below orf lesions differed phenotypically from the dendritic cells normally present in uninfected ovine skin; secondly, whether there was any relationship between these dendritic cells and cells of the monocyte-macrophage lineage; and thirdly, whether the dendritic cells were proliferating locally.

## MATERIALS AND METHODS

*Sheep:*

Female Suffolk cross sheep, reared at the Moredun Research Institute were used in all the experiments.

*Group 1. Primary orf virus infection.*

Four hysterectomy-derived specific pathogen-free (SPF) lambs were kept in an environment free from orf virus before and during the experiment. When two months old, all four animals were given a primary infection with orf virus following the method described below.

*Group 2. Secondary orf virus infection.*

Four conventionally reared sheep, aged between 12 and 14 months, were used. Blood samples were taken from the animals prior to the start of the experiment and analysis of the sera by ELISA (5) revealed positive titres to orf virus antigen which indicated previous exposure. The animals were challenged experimentally with orf virus as outlined below.

*Group 3. Control scarification.*

Four conventionally reared control sheep aged between 12 and 14 months were scarified experimentally but not challenged with orf virus.

***Viral inoculum***

The virus used in the experiment was Scabbymouth, a vaccine produced by the Commonwealth Serum Laboratory of Australia consisting of live tissue culture propagated orf virus which has retained virulence.

*Inoculation Procedure:*

All 12 animals were scarified on the inner thigh with a single stroke using a 16 gauge needle. To groups 1 and 2, 0.1ml of the vaccine ($10^7$ $TCID_{50}$/ml) was applied topically to the abraded skin. To the scarification lines of group 3, 0.1ml of phosphate buffered saline (PBS) was applied. Skin samples (6mm in diameter), were taken by punch biopsy from all 12 animals across the scar lines at 0h, 48h, 96h and 170h after treatment. Extra biopsies were collected from the primary infected animals at days 9, 12, 16, 22 and 30 for qualitative observations.

*Immunohistochemistry*

Each skin specimen was fixed for 2-3h at 4°C in a cacodylate/formalin solution (6), then stored for approximately 18h in 0.4M sucrose at 4°C, as described previously (4). One half of each biopsy was processed to paraffin wax by the St.Marie method (7) and the other half snap frozen in liquid nitrogen, then stored at -70°C. For quantitation of dendritic cells by immunohistology, five semi-serial paraffin wax sections, each 10μm thick, were cut from each sample at intervals of at least 50μm and mounted on slides pre-coated with Biobond (British Biocell, Cardiff, UK). Sections of frozen tissue were cut at 10μm thick in a cryostat for immunostaining with some of the antibodies (see below).

Using an avidin-biotin immunoperoxidase kit, (Vectastain elite, Vector laboratories Ltd, Burlinghame, USA) a set of slides from each sheep was immunostained with a panel of specific antibodies listed below. The sections were incubated with the panel of antibodies overnight at 4°C and developed following the kit instructions. Briefly, biotinylated horse IgG anti-mouse immunoglobulin was used as a second stage reagent, followed by a horse radish peroxidase-labelled avidin-biotin complex. For the rabbit antiserum against factor XIIIa, biotinylated goat anti-rabbit was used as the second stage reagent. Visualisation and location were achieved by diaminobenzidine

(DAB; Dako Ltd, High Wycombe). For 2 colour analysis a second monoclonal antibody (mab) was applied after the first had been coloured by DAB, followed by the

incubations described as above. The second antibody was labelled with alkaline phosphatase and revealed using a different coloured substrate, Vector New fuschin, which gives a pink colour. All slides were counterstained in haematoxylin, dehydrated through graded alcohols and histoclear (National Diagnostics, New Jersey) and mounted in histomount (National Diagnostics, New Jersey).

***Antibodies***

*Dendritic cell markers*

Two monoclonal antibodies (mabs) and one polyclonal antibody preparation were used for immunohistological characterisation of dendritic cells using previously optimised concentrations of each of the following: SBU-49.1, an anti-MHC class II antibody that recognises an epitope common to ovine DR and DQ (8); SBU-T6, an anti-ovine pan CD1-specific antibody (9,10) and rabbit IgG anti-human coagulation factor XIIIa (1:750 Calbiochem, La Jolla, USA).

*Detection of proliferation*

The mab to proliferating cell nuclear antigen (PCNA) clone PC10 (Dako, Ltd), was used to detect the cells undergoing proliferation. The antibody to PCNA recognises a phase-specific nuclear antigen that functions as a co-factor for DNA polymerase delta (11). PCNA levels are increased through the mid G1 phase of the cell cycle, remain elevated throughout the S phase, then rapidly decrease from G2/M to G1 (12)

*Monocyte-macrophage markers*

Three mabs reactive with monocyte-macrophages were used; OM-1 , anti-ovine CD11c (13); IL-A15, anti-ovine/bovine CD11b (14) and IL-A24, anti-ovine/bovine p110/75 antigen which reacts with a subpopulation of dendritic cells as well as neutrophils, eosinophils, macrophages and some lymphocytes (15).

*Orf virus*

The mab, 5E2, specific for an unidentified orf virus protein was used (H.W.Reid, Moredun Research Institute).

*Controls*

Negative controls included isotype-matched murine mabs specific for border disease viral epitopes (obtained from G. Entrican, Moredun Research Institute) and rabbit serum IgG as a control for the factor XIIIa-specific antibody.

Positive cell controls for the macrophage markers consisted of cytospins of ovine alveolar macrophages (prepared by G. Entrican, Moredun Research Institute) fixed in formaldehde/cacodylate as described above.

*Quantification of dendritic cells*

Dendritic cells, defined by previously reported criteria (16), were quantified within a total area of 1mm2 of dermis on five semi-serial sections of each biopsy for normal skin and at the time points 48h, 96h and 170h after treatment (4) for each of the three mabs used. The counts were performed on four microscope fields across each section of skin. Dual staining of dendritic cells with 2 of the 3 mabs was determined by comparison of serial sections or by the two colour technique described. Counts were not performed for the mab staining of cells other than dendritic cells or for the additional sections of primary orf lesions collected at timepoints beyond 170h.

## RESULTS

The results for each of the monoclonals are described individually and subdivided into four groups; normal skin, scarified skin, primary orf virus infection and secondary orf virus infection.

### *Dendritic cell phenotype.*

#### *Normal skin*

Four populations of phenotypically distinct MHC class II$^+$ dendritic cells were observed in normal skin; their phenotypes are summarised in Table 1. CD1$^+$ dendritic cells were seen within the epidermis generally at, or immediately above, the stratum basale and within the dermis clustered at the dermal/epidermal junction or closely associated with dermal structures such as sweat glands, hair follicles and blood vessels. A comparison between dendritic cells expressing MHC class II and CD1 antigens in serial sections (Figs 1a and 1b) showed that all CD1$^+$ dendritic cells coexpressed class II antigens. Within the epidermis all dendritic cells were CD1$^+$ and class II$^+$, but only 50% of the MHC class II$^+$ dendritic cells in the dermis were positive for CD1. A small population of factor XIIIa$^+$ dendritic cells were observed within the dermis, typically at the dermal/epidermal junction (Fig 1c) and surrounding blood vessels, particularly in the papillary dermis. Analysis of serial sections showed that factor XIIIa$^+$ dendritic cells were CD1$^-$ and coexpressed MHC class II. However, they represented only a small proportion (approximately 15%, Table 1) of the MHC class II$^+$ dendritic cells present within normal ovine skin.

#### *Scarified skin*

A significant increase of MHC class II$^+$ dendritic cells and factor XIIIa$^+$ dendritic cells from normal skin was observed at 48h following scarification ($p < 0.05$ and $p < 0.01$ respectively). These dendritic cell populations accumulated transiently at the dermal/epidermal junction on either side of the abrasion at 48h (Figs 2a, 3a and b). CD1$^+$ dendritic cells were absent from the dendritic cell accumulation and were found only in the undisturbed dermis/epidermis adjacent to the abrasion. At 96h CD1$^+$

dendritic cells had appeared along the boundary of the new epidermis and by 170h their distribution in the epidermis and dermis had returned to that seen in normal, uninfected skin.

*Primary Orf virus infection*

The dendritic cell response at 48h post-infection was similar to the response observed for scarification, with an increase in MHC class II$^+$ and factor XIIIa$^+$ dendritic cells. From 48h onwards the MHC class II$^+$ dendritic cells continued to accumulate forming a dense network beneath the infected, degenerating epidermis (see Figs 2a and 4a) which reached a maximum between days 9 to 12 and then decreased returning to a pre-infection distribution by day 30 (not shown). Factor XIIIa$^+$ dendritic cells accumulated in high numbers at the lesion (Figs 2b, 4b and 4c), forming a significantly high proportion (53%, $p < 0.0001$) of the dendritic cell network at 170h when compared with the equivalent time point in the scarification control group. Factor XIIIa$^+$ dendritic cells remained in the dermis beneath the lesion, unlike the MHC class II$^+$ dendritic cells which could be seen infiltrating the infected epidermis. Although 170h post-infection was the last timepoint for cell enumeration, qualitative analysis thereafter showed that factor XIIIa$^+$ cells persisted until about day 12 and then declined in parallel with the MHC class II$^+$ dendritic cells. The dense accumulation of dendritic cells was negative for CD1 at all the timepoints studied. With the onset and spread of infection (from 48h onwards), CD1$^+$ dendritic cell numbers decreased (Fig 2b), and by 170h these cells were virtually absent from the skin when compared with the same timepoint of scarified only skin ($p < 0.015$). On resolution of infection (day 16, as shown by disappearance of viral antigen) CD1$^+$ dendritic cells reappeared at the epidermal/dermal junction and subsequently within the thickened epidermis on day 22.

*Secondary orf virus challenge*

The MHC class II$^+$ dendritic cell response that occurred within the first 96h after a secondary challenge with orf virus (Fig 2c) was similar to the response observed during this time following primary infection. The dendritic cell network was densest at 96h after which cell numbers declined as the infection resolved. This is consistent with previous observations made by Jenkinson et al (4). Factor XIIIa$^+$ dendritic cells peaked transiently at 48h, as observed for scarification only controls, and were not present in the dense network of MHC class II$^+$ dendritic cells at 96h, representing only 1% of the total MHC class II$^+$ dendritic cellS present in the section. However, as the infection started to regress (170h) the numbers of factor XIIIa$^+$ dendritic cells returned to normal. CD1$^+$ dendritic cells were completely absent from the lesion and their numbers decreased as the lesion extended into previously unaffected dermis/epidermis (Fig 2c) At 170h CD1$^+$ dendritic cells were present at the dermal/epidermal junction of the healing lesion.

***Proliferating cell nuclear antigen (PCNA)***

*Normal skin*

PCNA$^+$ cells were present in low numbers in the normal skin of all the animals studied. Typically the positive cells were observed in the basal layer of the epidermis and surrounding sweat glands and at the base of hair follicles. These cells did not have a dendritic cell morphology.

*Scarified skin*

Intense PCNA staining was observed at 48h post scarification in the epidermal cells flanking the edges of the newly regenerating epidermis (Fig 5a). The cells infiltrating the dermis beneath the lesion were negative for PCNA. The newly formed epidermis also contained some positive cells at 96h, and by 170h the skin resembled the normal state, with only faintly PCNA$^+$ cells detectable.

*Primary orf virus infection*

At 48h post infection, PCNA staining was similar to that seen at the same time following scarification only, being focused at the edges of the lesion and in the newly formed epidermis. The proliferative activity within the epidermis decreased from 96h when the epidermal cells at the edges of the expanding lesion became vacuolated due to viral replication and only faint staining with PCNA was detectable. By 170h PCNA staining began to increase and intense nuclear staining was observed within the developing epidermal downgrowths (Fig 5b), which increased in size over the next few days (days 9 to 12, not shown). Positive cells also were present within the dermal cellular influx (48h onwards). Dual staining of these cells with MHC class II revealed that a few of the proliferating cells were class $II^+$ dendritic cells (Fig 5c). By days 22 to 30 $PCNA^+$ cell numbers decreased and the skin returned to a normal level of pre-infection PCNA staining.

*Secondary orf virus challenge*

The pattern of cellular proliferation was similar to the scarification already described, with positive cells flanking the edges of the lesion at 48h. Proliferation was not observed at any time point within the dermal influx of dendritic cells, the dense accumulation of cells at 96h was completely negative for PCNA staining. Epidermal downgrowths were not observed and the only proliferating cells that were present were within the healing epidermis (at 170h).

*Monocyte-macrophage markers*

All three mabs recognised markers on macrophages in the fixed cytospins of alveolar macrophages (not shown). Within normal skin, the occasional positive cell that had macrophage-like morphology (round nucleus, lack of marked dendritic processes) could be detected within the dermis with the antibodies OMI and IL-A15. These cells were associated typically with dermal structures such as sweat glands. However IL-A24 did recognise cells with dendritic processes in the epidermis and surrounding the tops of hair follicles. Some staining with the antibodies IL-A15 and IL-A24 of the

inflammatory influx generated in response to scarification, primary and secondary orf virus infections was observed, with the largest number of positive cells occuring in the primary orf lesion. Under higher magnification these positive cells were seen to be neutrophils. Neither IL-A24$^+$ dendritic cells nor macrophages was detected. OM1 did not stain any of the cells present within the inflammatory cell influx.

*Orf virus antigen*

Orf viral antigen was not detected in normal or scarified skin. In both primary and secondary infections viral antigen was present in cells adjacent to the damaged epidermis at 72h. In secondary challenge infections viral antigen was detected only up to 96h, whereas primary infections had more extensive staining and viral antigen was still detectable at day 22.

## DISCUSSION

Jenkinson et al (4) showed that the MHC class II$^+$ dendritic cells involved in the cutaneous response to orf virus infection share the phenotype of the dermal dendritic cells observed in normal ovine skin, characterised by the absence of the Langerhans cell marker AchE. They hypothesised that dermal dendritic cells were likely to represent a heterogeneous population of cells that may be differentially involved in the MHC class II$^+$ dendritic cell response to orf virus infection.

Our studies show that within normal ovine skin the MHC class II$^+$ dendritic cells comprise at least four subpopulations of dendritic cells; one is found exclusively within the epidermis- the Langerhans cell and is AchE$^+$ and CD1$^+$. The AchE- dermal dendritic cells described by Jenkinson et al (4) constitute three subpopulations; CD1$^+$ dendritic cells (that represent about 50% of the total) and CD1$^-$ dendritic cells of which a proportion (approximately 36%) are positive for the marker, factor XIIIa. This marker has been used recently to define human dermal dendritic cells of bone marrow origin (17, 18 and 19). The dendritic cells that gather in response to scarification and orf virus infection were a CD1$^-$ population comprising two of the subpopulations found within the dermis of normal ovine skin. The expression of factor XIIIa by these cells differed between primary and secondary orf virus infections.

In response to the trauma caused by scarification, factor XIIIa$^+$ dendritic cells increased transiently in number, gathered at the edges of the lesion and possibly play a role in the initial healing response. Studies in man have shown that factor XIIIa is found regularly in the early stages of repairing lesions, most clearly in evolving scars (20). Factor XIIIa is a transglutaminase involved in the final stages of clot formation (21) and one theory suggests that factor XIIIa is stored within cells for extracellular release after local injury, crosslinking the fibrin clot to structural protein and helping to seal the wound (22). In the present study, the diffuse non cell-associated staining often observed at the edges of the lesion and within the scab could be due to the deposition of factor XIIIa at the scar and may also explain its transient expression.

Factor XIIIa$^+$ dendritic cells were present in much higher numbers and persisted for much longer in the primary orf virus lesion compared with the scarified skin and the secondary orf lesion. This may have been due to the more extensive nature of the lesion, or these cells may have a role other than in wound healing. Recently human factor XIIIa$^+$ -dendritic cells have been identified as coexpressing intracellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen (LFA-1). The presence of these adhesion molecules and the distinctive distribution of these cells (with the highest concentration being in the skin and mucosal tissues) has led to the suggestion that they play a central role in skin immune responses (22). Factor XIIIa$^+$ dendritic cells have been demonstrated in psoriatic skin lesions as being potent producers of tumour necrosis factor -$\alpha$ (TNF$\alpha$) (23) which is implicated in the increased growth rate of the keratinocytes in the lesions. It was hypothesised that the ability of these dermal dendritic cells to produce TNF$\alpha$ could increase lymphocyte trafficking through the skin by inducing IL-8 and ICAM-1 production by keratinocytes (23). Thus a possible function of the cells could be the attraction of neutrophils and T lymphocytes into the skin in response to epidermal damage or viral replication. High numbers of factor XIIIa$^+$ dendritic cells are seen in the verrucous skin lesions of HIV-1$^+$ patients caused by secondary infections with DNA viruses, such as varicella zoster virus, herpes simplex virus and molluscum contagiosum (24). The ability of factor XIIIa$^+$ dendritic cells to influence keratinocyte growth could contribute to the process of healing damaged skin. Interestingly, factor XIIIa$^+$ dendritic cells were most numerous adjacent to the epidermal downgrowths which are characteristic of primary but not secondary orf lesions. As the secondary orf lesion heals well without the presence of these cells, the high numbers of factor XIIIa$^+$ dendritic cells that accumulate in a primary orf lesion may have an immunological role possibly reflecting fundamental differences in the primary and secondary immune response to orf virus infection, and contribute to the epidermal proliferation which characterises the primary lesion.

It is unknown whether the expression of the antigens CD1 and factor XIIIa can be

influenced by cytokines. The differences in the level of expression of factor XIIIa between primary and secondary responses could be explained by differences in the local cytokine profiles. The depletion of CD1 from the surrounding dermis may occur as a direct result of orf virus replication and the induction or suppression of local cytokines which may regulate CD1 expression. The levels of CD1 return to normal only when the infection is well into regression. Cerio et al (18) studied dendritic dermal cells that expressed factor XIIIa and concluded that they were bone marrow-derived, distinct from Langerhans cells and shared some common epitopes with mononuclear phagocytes. The relationship of the dendritic cell with the monocyte macrophage lineage is still under debate. The lack of staining of the MHC class $II^+$ dendritic cells with a panel of monoclonal antibodies which recognise markers on macrophages confirms only that these cells are not MHC class $II^+$ tissue macrophages but still leaves their origin unknown. It is possible that the network of MHC class $II^+$ and $CD1^-$ dendritic cells represent an immature population of dendritic cells. The maturation and subsequent expression of specific markers by these cells may be dependent upon the production of certain cytokines, which is affected by orf virus infection thereby maintaining the cells in an immature state.

As the accumulation of cells in response to scarification and secondary orf virus infection occurs in the absence of any measurable proliferative activity, it is unlikely that the small population of MHC class $II^+$, $CD1^-$ dendritic cells present in normal ovine skin proliferate locally to form the dense network of dendritic cells. The regenerating epidermal keratinocytes, positive for PCNA at 48h post-scarification and infection contain orf virus during the eclipse phase-when no viral antigen is detectable. From 96h onwards, these cells become vacuolated, $PCNA^-$ and contain orf virus antigen. High proliferative activity is however observed within the dermal influx of cells in primary orf virus infections. Dual staining revealed that a few of these proliferating cells are MHC class $II^+$ dendritic cells. A high proportion of the remaining proliferative activity is probably due to lymphocyte proliferation. The large accumulation of MHC class $II^+$ dendritic cells, most likely arises from an influx of cells from the blood. Whether this number represents recruitment of circulating

dendritic cells or a population of precursor cells that develop their dendritic characteristics after infiltrating the tissues is not known.

In summary, the dendritic cells accumulating in orf virus- infected skin share the phenotype of the $CD1^-$ population of dermal dendritic cells that are found in normal ovine skin. A proportion of these cells express the marker factor XIIIa, the number varying considerably between primary and secondary responses. Detailed functional studies are required to determine whether factor XIIIa and CD1 are inducible on the dendritic cells and if expression of the different markers reflects functional differences between the populations. The information gained from these studies should then provide an insight into the possible origin and role of the dendritic cell at the site of the orf virus lesion.

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Table 1.

| markers/cells | MHC class II | AchE | CD1 | factor XIIIa | %* |
|---|---|---|---|---|---|
| Epidermal DC | + | + | + | - | |
| Dermal DC (1) | + | - | + | - | 45 |
| Dermal DC (2) | + | - | - | + | 15 |
| Dermal DC (3) | + | - | - | - | 40 |

* Each population as a percentage of the total MHC class II$^+$ dendritic cells present within the dermis.

## Figure legends

**Table 1.** The dendritic cell populations of normal ovine skin.

**Figure 1.** Serial sections of normal ovine skin, showing The distribution of (a) MHC class II$^+$ dendritic cells, (b) CD1 dendritic cells and (c) factor XIIIa$^+$dendritic cells. **(x 560)**

**Figure 2.** Changes in the MHC class II$^+$, CD1$^+$and factor XIIIa$^+$ dendritic cell populations in response to;

(a) mock scarification

(b) primary orf virus infection

(c) secondary orf virus infection

**Figure 3.** Serial sections of ovine skin 48h post-scarification, showing the accumulation of; (a) MHC class II$^+$ dendritic cells and (b) factor XIIIa$^+$ dendritic cells at the dermal/epidermal junction of the abrasion. Note the diffuse staining at the edges of the lesion with factor XIIIa. **(x 560)**

**Figure 4.** Serial sections of ovine skin 170h post-primary infection with orf virus, showing the extensive nature of the lesion, with the balloning degeneration of the epidermis and associated hair follicles and the accumulation of ; (a) MHC class II$^+$ dendritic cells and (b) factor XIIIa$^+$ dendritic cells.**(x 112)**

(c) A high power view of the dendritic nature of the factor XIIIa$^+$ cells.**(x 560)**

**Figure 5.** The proliferative response of ovine skin

(a) 48h post-scarification, the epidermal keratinocytes involved in the healing process of the skin are PCNA positive. **(x 112)**

(b) 170h post-primary infection with orf virus. A large number of intensely PCNA$^+$ cells are seen in the developing epidermal downgrowths, surrounding infected hair follicles and within the dermal influx. **(x 112)**

(c) Double staining with MHC class II (pink) and PCNA (brown) shows a

proliferating MHC class $II^+$ dendritic cell within the inflammatory influx. Immunoperoxidase and alkaline phosphatase. **(x 560)**